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Detection of genome, antigen, and antibodies in oral fluids from pigs infected with foot-and-mouth disease virus

Chandrika Senthilkumaran, Ming Yang, Hilary Bittner, Aruna Ambagala, Oliver Lung, Jeffrey Zimmerman, Luis G. Giménez-Lirola, Charles Nfon

Abstract

Virus nucleic acids and antibody response to pathogens can be measured using swine oral fluids (OFS). Detection of foot-and-mouth disease virus (FMDV) genome in swine OFS has previously been demonstrated. Virus isolation and viral antigen detection are additional confirmatory assays for diagnosing FMDV, but these methods have not been evaluated using swine OFS. The objectives of this study were to further validate the molecular detection of FMDV in oral fluids, evaluate antigen detection and FMDV isolation from swine OFS, and develop an assay for isotypic anti-FMDV antibody detection in OFS. Ribonucleic acid (RNA) from FMDV was detected in OFS from experimentally infected pigs by quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR) from 1 day post-infection (dpi) to 21 dpi. Foot-and-mouth disease virus (FMDV) was isolated from OFS at 1 to 5 dpi. Additionally, FMDV antigens were detected in OFS from 1 to 6 dpi using a lateral flow immunochromatographic strip test (LFIST), which is a rapid pen-side test, and from 2 to 3 dpi using a double-antibody sandwich enzyme-linked immunosorbent assay (DAS ELISA). Furthermore, FMDV-specific immunoglobulin A (IgA) was detected in OFS using an isotype-specific indirect ELISA starting at dpi 14. These results further demonstrated the potential use of oral fluids for detecting FMDV genome, live virus, and viral antigens, as well as for quantifying mucosal IgA antibody response.

Résumé

Chez les porcs les acides nucléiques viraux et la production d’anticorps contre des agents pathogènes peuvent être mesurés en utilisant les fluides oraux (FO). La détection du génome du virus de la fièvre aphteuse (VFA) dans les FO de porcs a été démontrée précédemment. L’isolement viral et la détection d’antigènes virales sont des épreuves de confirmation supplémentaires pour diagnostiquer la présence du VFA, mais ces méthodes n’ont pas été évaluées en utilisant des FO porcins. Les objectifs de la présente étude étaient de valider un peu plus la détection moléculaire du VFA dans les FO, d’évaluer la détection d’antigènes et l’isolement du VFA à partir de FO porcins, et de développer une épreuve pour la détection d’anticorps isotypiques anti-VFA dans les FO. L’ARN du VFA fut détecté dans les FO de porcs infectés expérimentalement par réaction quantitative en temps réel d’amplification en chaîne par la polymérase utilisant la transcriptrase réverse à partir du jour 1 post-infection (PI) jusqu’au jour 21 PI. Le VFA fut isolé à partir des FO aux jours 1 à 5 PI. De plus, les antigènes du VFA ont été détectés dans les FO des 1 à 6 PI en utilisant une épreuve sur bandelette d’immunochromatographie par flot latéral, un test rapide pouvant être réalisé à la ferme, ainsi que de 2 à 3 j PI en utilisant une épreuve immuno-enzymatique (ELISA) double-sandwich. Également, à partir du jour 14 PI des immunoglobulines A (IgA) spécifiques au VFA ont été détectées dans les FO au moyen d’une épreuve ELISA indirecte spécifique pour les isotypes. Ces résultats démontrent d’une manière additionnelle le potentiel d’utilisation des FO pour détecter le génome du VFA, du virus vivant, et des antigènes viraux, de même que pour quantifier la production d’IgA par les muqueuses.

(Traduit par Docteur Serge Messier)
Oral fluids, or saliva, have been used in human medicine to diagnose infectious and metabolic diseases and hormonal disorders since 1957 (8,9). The use of OFs in veterinary medicine has become popular over the last 2 decades (10). Several recent studies in pigs have shown that OFs can be used to diagnose highly infectious viral diseases and for disease surveillance (10–15).

While the use of swine OFs for FMDV detection has previously been reported (14,15), these studies focused on detection of the FMDV genome by real-time reverse transcription-polymerase chain reaction (qRT-PCR). Other assays based on the use of swine OFs to detect FMD have not been evaluated. Therefore, the objectives of this study were to further validate the use of swine OFs for the detection of FMDV genomes and to evaluate FMDV antigen detection in OFs using a double-antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA) and a rapid lateral flow immunochromatographic strip test (LFIST). An additional objective was to develop an ELISA for detecting anti-FMDV immunoglobulin A (IgA) in oral fluids from pigs experimentally infected with FMDV.

**Materials and methods**

**Experimental design**

The Animal Care Committee at the Canadian Science Centre for Animal and Human Health reviewed and approved the use of animals for this study under animal-use document (AUD) number 15-001. The Canadian Council for Animal Care guidelines were observed during all procedures with animals.

Twenty-four Landrace 5- to 6-week-old grower pigs were purchased from a local source in Manitoba, Canada. Upon arrival at the National Centre for Foreign Animal Disease (NCFAD), the pigs were randomly assigned into 4 groups (A to D) of 6 pigs each and housed in 4 separate containment-level 3 animal cubicles. The pigs were allowed 7 d to acclimatize to their new surroundings before virus inoculation. Food and water were provided ad libitum and the virus inoculation. Food and water were provided ad libitum and thereafter until the end of the experiment at dpi 28, as previously described (17).

**Quantitative real-time reverse transcription-polymerase chain reaction (qRT-PCR)**

The amount of viral ribonucleic acid (RNA) in oral fluids, oral swabs, nasal swabs, and serum samples was quantified by a TaqMan qRT-PCR assay that specifically amplified an 88-bp conserved region of the FMDV 3D gene. Total RNA was extracted from the samples using the MagMAX-96 Viral RNA Isolation Kit (AMB1836-5; Life Technologies, Burlington, Ontario) and the MagMAX Express-96 Magnetic Particle Processor (Life Technologies), following the manufacturer’s protocol. One-step qRT-PCR was carried out using AgPath ID One-Step RT-PCR reagents (AM1005; Life Technologies) on the Applied Biosystems 7500 Real-Time PCR Instrument (Life Technologies) as previously described (18). To determine diagnostic specificity of the qRT-PCR for oral fluids (OFs), 300 OF samples collected from groups of clinically healthy and FMDV-free pigs in Canada and the US were tested.

**Virus isolation and titration**

Virus was isolated from oral fluids and oral swab samples on monolayers of a fetal porcine kidney cell line constitutively expressing α,β, integrin (LFBK, α,β, cells) (19,20), using a previously described method (17).

**Detection of antigen by double-antibody sandwich ELISA (DAS ELISA)**

The presence of viral antigen in oral swabs, oral fluids, and sera was tested by a double-antibody sandwich (DAS) ELISA, as previously described (16,21).

**Detection of FMDV antigen by the lateral flow immunochromatographic strip test**

Foot-and-mouth disease virus (FMDV) serotype O antigen was detected in oral fluids (OFs) by a lateral flow immunochromatographic strip test (LFIST) described previously (22). Briefly, 50 μL of OFs was mixed with a 50-μL cocktail of capture and colloid-gold-conjugated detection monoclonal antibodies in a running buffer (RapidAssays ApS, Copenhagen, Denmark). If FMDV antigen was present, immune complexes were formed between the antigen and the monoclonal antibodies. The strips were dipped into this mixture, which then migrated along the membrane to the test and control lines. The results were interpreted as previously described (22).

**Detection of FMDV serotype O-specific IgA Isotype antibodies in oral fluids**

A modification of a previously described FMDV serotype O solid-phase competitive ELISA was used to detect FMDV serotype O-specific IgA (16,21). Briefly, 96-well ELISA plates (Nunc Maxisorb; Thermo Fisher Scientific, Waltham, Massachusetts, USA) were coated with a rabbit anti-FMDV O antibody in carbonate buffer, pH 9.6 (50 μL/well) and incubated overnight at 4°C. The contents of each well were emptied and the plates were blocked with 10% horse serum in PBST (phosphate-buffered saline, 0.05% Tween-20, pH 7.2) for 1 h at 37°C with shaking. After washing 5 times with
PBST, FMDV serotype O antigen in blocking buffer (50 μL/well) was added to the plates and incubated for 1 h at 37°C with shaking. The plates were washed 5 times and oral fluids diluted 1/2 in PBST containing 5% horse serum were added to the plates at 50 μL/well. After 1 h of incubation, plates were washed and mouse anti-pig IgA (AbD Serotec, Raleigh, North Carolina, USA) at approximately 0.17 μg/mL in PBST containing 5% horse serum was added to the plates (50 μL/well) and incubated for 1 h at 37°C with shaking. The plates were washed 5 times and tetramethylbenzidine (TMB; Thermo Scientific, Rockford, Illinois, USA) substrate was added to the plates (50 μL/well). The plates were incubated in the dark at room temperature for 10 min. After adding the stop solution [2 M sulfuric acid (H₂SO₄)], the optical density (OD) was read at 450 nm (OD₄₅₀) using an ELISA plate reader (SpectraMax Plus 384 Microplate Reader; Molecular Devices, Sunnyvale, California, USA). To determine diagnostic specificity, 299 OF samples collected from groups of clinically healthy and FMDV-free pigs in Canada and the US were tested on the same assay. In addition, oral fluids from pigs infected with swine vesicular disease virus (SVDV) and vesicular stomatitis virus (VSV) were tested to determine analytical specificity.

**Data analysis**

Receiver operating characteristic (ROC) analysis was conducted using Version 1.3 of easyROC (23). Results for known negative OF samples and from inoculated animals starting at dpi 14, 21, and 28, which allowed the humoral response to develop, were used for the ROC analysis. The optimal cutoff was determined by the Youden method (24).

**Clinical scores in pigs inoculated with foot-and-mouth disease virus**

Seven of the 8 pigs inoculated on the heel bulb developed fever and lesions typical of FMD at dpi 2. One directly inoculated pig died abruptly at dpi 2 without developing fever. Furthermore, 1 direct-contact animal also died abruptly without showing any clinical signs. At postmortem, necrotic myocarditis was observed in both pigs. The rest of the direct-contact animals developed fever and clinical
Table I. Detection of foot-and-mouth disease virus (FMDV) by real-time reverse transcription polymerase chain reaction (qRT-PCR). Five- to 6-week-old pigs were inoculated with FMDV and oral fluid (OF) samples were collected from each of 4 groups using cotton ropes; sera, oral swabs, and nasal swabs were also collected from individual animals. All samples were tested by FMDV 3D qRT-PCR. Crossing threshold (Ct) of < 35.99 was considered positive for FMDV genome. The lower the Ct value, the higher the amount of FMDV ribonucleic acid (RNA). Any sample with undetermined Ct was assigned a Ct of 40. Numbers in bold are positive for FMDV genome. The first 2 pigs in each group were directly inoculated.

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dpi — Days post-inoculation.
disease at dpi 3 and 4, 24 to 48 h later than the directly inoculated pigs (Figure 1). On or before dpi 7, most of the pigs (66.7%) were either dead or euthanized for humane reasons, due to severe lesions and/or lameness.

**Detection of FMDV in oral fluids and nasal swabs by qRT-PCR and virus isolation**

Ribonucleic acid (RNA) of foot-and-mouth disease (FMDV) was detected in oral fluids (OFs) at dpi 1 in groups A, B, and D and at dpi 2 in group C and preceded the onset of clinical signs. Ribonucleic acid (RNA) of FMDV was also detected in oral swabs and nasal swabs, starting at dpi 2 for most of the directly inoculated pigs and at dpi 3 for the direct-contact pigs. Similarly, based on FMDV RNA detection in serum, viremia began at dpi 1 in directly inoculated pigs and at dpi 2 to 3 in the direct-contact pigs (Table I). Higher levels of FMDV genome were detected in oral fluids than in oral swabs and nasal swabs at corresponding days post-infection (Figure 2). Additionally, there was prolonged detection of FMDV RNA in oral fluids (up to dpi 14 and 21), whereas only 2 samples from oral swabs were positive at dpi 14 and all nasal swabs and sera were negative after dpi 7 (Figure 2 and Table I). All 300 OF samples collected from farms in Canada and the US were negative, giving a diagnostic specificity of 100%.

Additionally, live FMDV was isolated from OF samples at dpi 1 to 5 in all the groups except group A, in which virus isolation started at dpi 2 (Figure 3). Virus isolation from oral swabs was delayed, starting at dpi 4 for most animals. Virus titers were higher in oral fluids than in oral swabs (Figure 3).

**Detection of antigen in oral fluids**

Foot-and-mouth disease virus (FMDV) antigen was detected in OF samples from groups A and B at dpi 2 and at dpi 3 in all 4 groups by the DAS ELISA (Figure 4). On the other hand, 1 sample from oral swabs on dpi 2, 3, and 5 and 5 oral swabs at dpi 4 were positive for FMDV antigen (data not shown).

Similarly, FMDV antigen was detected by LFIST at dpi 2 to 5 in OF samples from all 4 groups. The OF samples became negative for FMDV antigen at dpi 6 with the exception of group C that remained weakly positive.

**Detection of IgA antibody in oral fluids**

An optimal positive cutoff of OD ≥ 0.75 for anti-FMDV IgA in oral fluids (OFs) was determined by the Youden method (24). The distribution (scatter grams) of all the negative and positive samples is shown in Figure 5A. Diagnostic sensitivity and specificity were both 100%. The OF sample became positive for anti-FMDV IgA by DPI 14 (Figure 5B). There was no cross-reactivity with OF samples positive for antibodies to swine vesicular disease virus (SVDV) and vesicular stomatitis virus (VSV).
Collection of oral fluids (OFs) is cost-effective, stress-free for the animals, and has been proposed as an appropriate type of sample for disease surveillance (10,12,15). Testing oral fluids for the early detection of reportable diseases like FMD could therefore be highly beneficial to the pork industry around the world. The objective of this study was to evaluate the potential use of OFs as an alternate type of sample for detecting FMDV in experimentally infected pigs. Similar work has been reported by other groups (14,15). However, in addition to virus genome detection in these studies, we evaluated virus isolation and viral antigen detection by a double-antibody sandwich enzyme-linked immunosorbent assay (DAS ELISA) and a lateral flow immunochromatographic strip test (LFIST), which is a rapid pen-side test. Furthermore, we demonstrated the suitability of OF samples for detecting anti-FMDV antibodies (IgA) post-infection.

In this study, directly inoculated pigs developed viremia by dpi 1 and clinical signs by dpi 2, but these were delayed by 24 to 48 h in direct contacts, which is consistent with previous findings in pigs (24–26). The detection of FMDV genome in OFs from groups of FMDV-infected pigs preceded the onset of clinical signs, which is similar to previous findings (14,15). This is significant in that the presence of FMDV in an apparently healthy herd can be detected using swine OFs. In the event of an outbreak, OFs preemptively

Figure 3. Isolation of foot-and-mouth disease virus (FMDV) from oral fluids (OF) and oral swabs (OS). Five- to 6-week-old pigs were inoculated with FMDV and oral fluids were collected from each of 4 groups using cotton ropes; oral swabs (OS) were also collected from individual animals. Error bars represent standard deviation of mean. *Time points when pigs were less interested in ropes, which reduced amount of OF.

Figure 4. Detection of foot-and-mouth disease virus (FMDV) antigen in oral fluids (OF) by the double-antibody sandwich (DAS) ELISA. Five- to 6-week-old pigs were inoculated with FMDV and oral fluids were collected from each of 4 groups using cotton ropes and tested for FMDV antigen by a DAS ELISA. Samples with an optical density (OD) of > 0.1 were considered positive for FMDV antigen. Grp = group.
sampled from apparently healthy pigs within a radius of the outbreak could be tested by qRT-PCR and rapid control measures implemented before the appearance of clinical signs.

We reported the detection of SVDV in oral fluids from groups of pigs with subclinical disease (17). Similar to our previous report for SVDV (17), oral fluids are better than oral swabs and nasal swabs for detecting FMDV. This could be attributed to prolonged chewing on ropes (20 to 30 min), which enhanced the contents of oral fluids. In a previous study (15), the quantification cycles (Cq) for detecting FMDV RNA were lower for oral fluids (OFs) than for oral swabs, which indicates that levels of FMDV genome are higher in OFs than in oral swabs. In addition, Vosloo et al (14) reported the detection of FMDV genome in OFs but not in oral swabs in a group of pigs challenged with FMDV serotype A. Nevertheless, they also reported contrary results for a specific group of pigs challenged with FMDV serotype O (14) in which oral swabs (OSs) were positive, while samples from oral fluids (OFs) were negative for FMDV serotype O genome at some sampling points. The possibility was raised that proteolytic enzymes might have affected virus recovery in OFs due to the 30-minute collection time at room temperature. This does not appear to be the case, however, since the oral fluids (OFs) in our study were collected in a similar manner to that in another study (15).

Virus isolation is considered a confirmatory test for FMDV. Isolating FMDV from OFs as early as dpi 1 therefore adds value to the use of this type of sample for diagnosing foot-and-mouth disease (FMD). Detmer et al (28), however, isolated virus from nasal swabs and not from oral fluids (OFs) from pigs infected with FMDV serotype A. This could be explained by the fact that, during sampling of oral fluids, the ropes were left in the pen for 6 or 12 h rather than for the standard 20 to 30 min (28). Another possibility is that the virus on the ropes was inactivated since influenza A virus becomes inactivated on highly porous surfaces in less than 12 h (29).

Some laboratories, especially in FMD-endemic countries, are not equipped for qRT-PCR testing. On the other hand, ELISA can be carried out in most laboratories worldwide. Thus, the detection of FMDV antigen in OFs by DAS ELISA means that most laboratories can use this type of sample to diagnose FMD. Although the DAS ELISA is considered relatively less sensitive than qRT-PCR, it is one of the routine diagnostic tests discussed in the OIE Manual of Diagnostic Tests and Vaccines for Terrestrial Animals (30).

Combining the ease of collecting oral fluids (OFs) with a rapid pen-side test would simplify detection of FMD in pigs. The lateral flow immunochromatographic strip test (LFIST) is rapid, easy to perform, and has been used to diagnose FMD (22). We have shown in this study that LFIST detects FMDV antigen in oral fluids (OFs), meaning a pig farmer or attendant can easily collect OFs, test by LFIST, and make a preliminary diagnosis of FMD. This is significant since effective control of outbreaks and eradication of FMD depend on rapid detection. Furthermore, we had previously shown that the FMDV genome could be detected in OFs from infected pigs with a rapid field-deployable reverse-transcription insulated isothermal (RT-iiPCR) assay (31).

A previous study also explored mucosal antibody response in FMDV-infected pigs using saliva and reported the detection of FMDV-specific IgA, but not IgG and IgM (27). Therefore, in this study we focused on the detection of IgA in OFs, corroborating previous observations on saliva (26). The detection of anti-FMDV IgA in oral fluids (OFs) confirms the usefulness of this type of sample for serological diagnosis of FMD in pigs by the IgA ELISA.

In conclusion, foot-and-mouth disease virus (FMDV) and antibodies to FMDV can be detected in oral fluids (OFs) from FMDV-infected pigs. This confirms the relevance of oral fluids from swine for routine virological surveillance as well as the potential for using OFs for serological surveillance of swine herds for FMDV.

Acknowledgments

The authors thank the animal care staff at the National Centre for Foreign Animal Disease (NCFAD) for their support with animal experiments.
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References


Application of direct polymerase chain reaction assays for Campylobacter fetus subsp. venerealis and Tritrichomonas foetus to screen preputial samples from breeding bulls in cow-calf herds in western Canada

Cheryl L. Waldner, Sarah Parker, Karen M. Gesy, Taryn Waugh, Emily Lanigan, John R. Campbell

Abstract

The primary objectives of this study were to estimate the prevalence of Campylobacter fetus subsp. venerealis (Cfv) and Tritrichomonas foetus in breeding bulls from a sentinel cohort of cow-calf herds in western Canada and to estimate the association between positive test status and non-pregnancy. The final objective was to evaluate the application of these tests when: i) screening bulls in the absence of a recognized problem with reproductive performance, and ii) testing for diagnosis of poor pregnancy rates. The crude apparent bull prevalence for Cfv was 1.1% [95% confidence interval (CI): 0.5% to 2.1%; 8/735] and herd prevalence was 2.6% (95% CI: 0.3% to 9.0%; 2/78). The crude apparent bull prevalence for T. foetus was < 0.001% (95% CI: 0.0% to 0.5%; 0/735) and herd prevalence was < 0.001% (95% CI: 0.0% to 4.6%; 0/78). Cows from herds where at least 1 bull was test positive for Cfv were 2.35 times more likely (95% CI: 1.01% to 5.48%; P = 0.047) to not be pregnant than those with no positive bulls. Polymerase chain reaction (PCR) testing of preputial material collected into phosphate-buffered saline (PBS) was recommended for screening for T. foetus when the pre-test probability of infection was > 1%. The same test for Cfv was not recommended for screening moderate- and low-risk herds due to the high risk of false positives. Tests for both T. foetus and Cfv can be used to investigate herds with reproductive problems when also ruling out other risk factors. Regardless of the type of test used, however, 3 negative tests are required to rule out infection in high-risk situations.

Résumé

Les objectifs primaires de la présente étude étaient d’estimer la prévalence de Campylobacter fetus subsp. venerealis (Cfv) et Tritrichomonas foetus chez des taureaux reproducteurs d’une cohorte sentinelle issue de troupeaux vache-veau dans l’ouest canadien et d’estimer l’association entre un test positif et la non-gestation. L’objectif final était d’évaluer l’application de ces tests lors de : i) vérification des taureaux en absence d’un problème reconnu avec les performances de reproduction, et ii) épreuve diagnostique en présence de faibles taux de gestation. La prévalence apparente brute des taureaux pour Cfv était de 1,1 % [intervalle de confiance (IC) 95 % : 0,5 % à 2,1 %; 8/735] et la prévalence pour les troupeaux était de 2,6 % (IC 95 % : 0,3 % à 9,0 %; 2/78). La prévalence apparente brute des taureaux pour T. foetus était < 0,001 % (IC 95 % : 0,0 % à 0,5 %; 0/735) et la prévalence pour les troupeaux était < 0,001 % (IC 95 % 0,0 % à 4,6 %; 0/78). Les vaches provenant de troupeaux où au moins un taureau s’était avéré positif pour Cfv étaient 2,35 fois plus susceptibles (IC 95 % : 1,01 à 5,48; P = 0,047) de ne pas être gestante que celles provenant de troupeaux sans aucun taureau positif. L’analyse par réaction d’amplification en chaine par la polymérase de matériel prépuéal prélevé dans de la saline tamponnée était recommandée pour vérifier la présence de T. foetus lorsque la probabilité d’infection pré-test était > 1 %. Le même type d’analyse pour Cfv n’était pas recommandé pour la vérification des troupeaux à risque modéré et faible étant donné le risque élevé de faux positifs. Les tests pour T. foetus et Cfv peuvent être utilisés pour investiguer les troupeaux avec des problèmes de reproduction en même temps que les autres facteurs de risque sont éliminés. Toutefois, indépendamment du type de test utilisé trois tests négatifs sont requis pour éliminer la possibilité de l’infection dans les situations à risque élevé.

Introduction

Campylobacter fetus subsp. venerealis (Cfv) and Tritrichomonas foetus (T. foetus) are important causes of reproductive loss in herds that use natural breeding. While individual case reports have documented costly herd outbreaks in western Canada (1), to date no studies have investigated the frequency or prevalence of T. foetus and Cfv infection in beef bulls and cow-calf herds in western Canada.

The clinical presentation of these venereal pathogens is very similar and both pathogens should be considered when investigating herds with poor fertility (2). Chronically infected bulls are an important reservoir in maintaining and transmitting both T. foetus and Cfv. A common option for controlling these infections is testing, identification, and culling of infected bulls (2).

While recent studies have measured the performance of diagnostic tests for Cfv and T. foetus (2–7), there are important differences in the
availability, sensitivity, and specificity of tests for these 2 venereal pathogens. In addition to culture for *T. foetus*, which is available at many local clinics and laboratories, the use of polymerase chain reaction (PCR) tests for *T. foetus* is encouraged for both diagnosis and screening because of the reported improved sensitivity and specificity relative to culture (2,7,8). Culture of *Cf* is more technically demanding, however, and is available only at some diagnostic laboratories. Culture of *Cf* also has very low sensitivity when transport times to the lab exceed 24 h (2,9). Practical PCR tests for use on field samples for *Cf* that require extended transport times have only recently become commercially available (3,5,10). The performance of PCR tests for *Cf* and *T. foetus* differ (4–6), which raises questions about the relative interpretation and application of these tests in clinical practice.

The primary objectives of this study were to estimate the prevalence of *Cf* and *T. foetus* in breeding bulls from a sentinel cohort of cow-calf herds in western Canada and to estimate the association between positive test status and the risk of non-pregnancy. The final objective was to evaluate various risk scenarios for the application of these tests when screening bulls: i) in the absence of a recognized problem with reproductive performance, and ii) when testing for diagnosis of poor pregnancy rates.

### Materials and methods

In collaboration with local veterinarians across western Canada, cow-calf herd owners were recruited in 2014 and 2015 for a multi-year surveillance initiative. The herds were selected from various geographic regions in Alberta, Saskatchewan, and Manitoba to represent the distribution of herd sizes and density described in the 2011 Census of Agriculture data. Participants in the western Canadian Cow-Calf Surveillance Network were given the option to have their mature breeding bulls tested for infection with *Cf* and *T. foetus* before the 2015 breeding season. Local veterinarians were paid up to $500 to cover additional costs associated with collecting and shipping samples from each herd. The costs for laboratory testing were also paid by the study.

### Bull selection

Sampling for laboratory testing from mature bulls was offered to all participating herds as an extension of annual breeding soundness evaluations in the spring of 2015. Virgin bulls, either yearlings or virgin 2-year-olds, were not included in the study as infection with *T. foetus* or *Cf* was considered to be very unlikely in these animals; therefore, the predictive value of a single positive sample would be very low as would the cost effectiveness of testing.

### Sample collection

A single preputial sample was used to test for both *T. foetus* and *Cf*. The preputial cavity was scraped repeatedly (at least 10 times) with an individually wrapped 50-cm Al pipette attached to a 20-cc syringe, with suction applied during scraping. The pipette was then flushed into a screw-top plastic vial containing 2.0 mL of phosphate-buffered saline (PBS) solution. As soon as possible after collection, the inoculated vial was placed in a refrigerator if the sample was shipped the same day or in a −20°C freezer if there was an expected delay in shipping. Samples were shipped in an insulated container with a frozen icepack to the Western College of Veterinary Medicine, Saskatoon, Saskatchewan.

Veterinarians were asked to complete a sample submission form requesting baseline information on each bull, including animal identification, age, and breed. Producers were also asked whether the bull was exposed to communal grazing in the previous breeding season and was semen tested by a veterinarian in 2015.

On arrival at the Western College of Veterinary Medicine, the preputial samples were thoroughly mixed and aliquoted for testing for *Cf* within the research lab (KG, TW). The second aliquot was submitted to a commercial laboratory (Prairie Diagnostic Services (PDS), Saskatoon, Saskatchewan) for *T. foetus* testing. Submissions containing a positive *Cf* sample based on results from the research laboratory were also submitted to the commercial laboratory (PDS) for second-laboratory confirmation using the same primer set described for the research laboratory.

### Real-time PCR assay for *Cf*

Deoxyribonucleic acid (DNA) was released from 200 μL of prepuce scrapings in PBS [20 mM phosphate, 150 mM sodium chloride (NaCl)] using a direct heat lysis with minor alterations (3,5). The preputial pellet was re-suspended in 100 μL of sterile water before heating, followed by a 1:10 dilution in sterile water before analysis (3,5). The real-time polymerase chain reaction (qPCR) mixture was created using SYBR green (iQ SYBR Green Supermix; Bio-Rad, Mississauga, Ontario), 400 nM of each primer, and 2 μL of dilute lysate in a final volume of 25 μL. All samples were run in duplicate on a thermocycler (iCycler/MyIQ; Bio-Rad) as previously described (3,5) using a primer set targeting *Cf* (VenSF and VenSR) (11). Each test included no template and positive controls, also in duplicate. Melt-curve analysis was used to indicate infection status; the lower detection limit was 103 copies. The resulting data were analyzed using commercial software (iQ5 Optical System Software; Bio-Rad). Samples with a melt curvature signature comparable to the positive control, peak signal of 78.5°C ± 0.5°C [mean ± standard deviation (SD)], and threshold cycle (Ct) value of < 35 were considered positive.

The clinical sensitivity and specificity of the qPCR assay for *Cf* has previously been reported for preputial samples collected directly into PBS (5). The crude sensitivity estimates from a group of 8 *Cf*-positive samples collected repeatedly was 85.4% (95% CI: 80.6% to 89.2%; 222/260). Based on testing of 300 virgin bulls, the crude clinical specificity estimates for the qPCR assay were 85.0% (95% C: 80.5% to 88.6%; 255/300).

### Real-time PCR for *T. foetus*

Direct preputial samples were tested with qPCR at a commercial diagnostic laboratory (Prairie Diagnostic Services, Saskatoon, Saskatchewan). DNA was extracted from a 200 mL aliquot of medium using a commercial kit (DNeasy Blood & Tissue BioSprint 96 One-For-All Vet Kit; Qiagen, Mississauga, Ontario). The qPCR assay was carried out using a Stratagene Mx3005P QPCR (Agilent Technologies Canada, Mississauga, Ontario), as described in a previous study (12). Samples were considered positive if the Ct value was < 40 (6).

Sensitivity of qPCR for *T. foetus* preputial samples collected directly into PBS was previously reported to be 90.1% (95% CI: 83.5%
to 94.2%; 109/121) for this laboratory (6). Although clinical specificity has not been reported for samples collected directly into PBS, the specificity of the assay from this laboratory for qPCR testing of samples collected in InPouch TF pouches (Biomed Diagnostics, San José, California, USA) was 100% (95% CI: 98.9% to 100%; 337/337) (4).

**Reporting**

The laboratory results for each herd were sent to the submitting veterinarian to share with the herd owner. Any herds that had a positive sample for *Cfv* or *T. foetus* were offered the choice of retesting their bulls.

A questionnaire was sent to producers in the fall of 2015 regarding conception outcomes for cows and heifers that were bred in 2014 to calve in 2015. Producers were asked to indicate if they only checked a portion of their animals, and if so, to estimate the open rate for both cows and heifers. Only the herds in which all cows and heifers were pregnancy tested were considered in the analysis of the association between infection status and pregnancy outcomes.

A questionnaire sent to all participants in the spring of 2014 provided baseline data on herd size and management. The requested information included whether a veterinarian had examined the bulls before the 2013 breeding season and whether the bulls were tested for *T. foetus* in the spring of 2013.

### Statistical analysis

The participating herds and sampled bulls were described, as well as the pregnancy rates for the subset of herds in which all cows and heifers were pregnancy tested. A bull was considered test positive for either *Cfv* or *T. foetus* if the PCR test was positive at least once.

### Table I. Characteristics of the sample population (n = 735 bulls, N = 78 herds)

<table>
<thead>
<tr>
<th>Attributes</th>
<th>Percent of bulls (n)</th>
<th>Percent of herds (N)</th>
<th>Median (IQR)</th>
<th>Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bull age (y)</td>
<td></td>
<td>4 (3, 5)</td>
<td>2 to 13</td>
<td>(n = 690)</td>
</tr>
<tr>
<td>Province</td>
<td></td>
<td></td>
<td></td>
<td>(n = 690)</td>
</tr>
<tr>
<td>Manitoba</td>
<td>19% (15)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Saskatchewan</td>
<td>27% (21)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Alberta</td>
<td>53% (41)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>British Columbia</td>
<td>1% (1)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Breed</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Angus</td>
<td>27% (272)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Simmental</td>
<td>22% (158)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Charolais</td>
<td>9% (67)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gelbvieh</td>
<td>9% (66)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Limousin</td>
<td>5% (39)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hereford</td>
<td>4% (32)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Other</td>
<td>8% (64)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Not reported</td>
<td>5% (39)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Exposure to communal grazing</td>
<td>10% (73)</td>
<td>28% (22)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bulls semen tested by a</td>
<td>98% (717)</td>
<td>99% (77)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>veterinarian in current year</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bulls semen tested by a</td>
<td>90% (70)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>veterinarian in previous year</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bulls tested for <em>T. foetus</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>in previous year (2014)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>21% (16)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Unsure</td>
<td>8% (6)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

IQR — Interquartile range.
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herd was considered test positive if at least 2 bulls were test positive for Cfv or at least 1 bull was test positive for T. foetus. The difference in the number of reactors required for a herd to test positive was influenced by the lower specificity of the PCR test for Cfv.

The association between having at least 2 bulls that tested positive for Cfv or at least 1 bull that tested positive for T. foetus and the risk of non-pregnancy in the herds that pregnancy tested all of their females exposed to breeding was examined using generalized estimating equations. The data were examined using a negative binomial distribution with a log-link function and a robust variance estimate, while accounting for clustering of pregnancy outcomes within herd. The outcome variable was the total count of non-pregnant females. The respective offsets were the log of the total number of cows and heifers pregnancy tested. The difference in the risk of non-pregnancy between heifers and cows was assessed in this model and all subsequent models were stratified for heifers and cows.

As they could be potential confounders, other management risk factors considered in the models included: if the herd was exposed to community pasture during the summer of 2014; if the herd had more than 300 cows at calving in 2014; the month of first bull exposure in 2014; whether calving started before March 2014; if the calving season was more than 4 cycles or 84 d; and whether or not the herd sold at least some purebred cattle. Models were built using manual backwards stepwise removal. Only factors that were significantly associated with the risk of non-pregnancy or were not significant but were important confounders were retained in the final model. Factors that changed the regression coefficient for infection status by more than 20% when added or removed from the model were considered important confounders. Biologically plausible 2-way interactions were assessed among variables retained in the final model. The difference in non-pregnancy risk between test-positive and test-negative herds was reported as a risk ratio (RR) with 95% confidence intervals (95% CI).

Apparent prevalence was reported with 95% CI (13) for both the proportion of test-positive bulls and positive herds. The true individual prevalence of infection with 95% CI was simulated from Table II. Summary of available pregnancy test data for fall 2014 from 72 of the 78 herd owners responding to a survey and effect estimates for herd management factors that could be associated with risk of non-pregnancy

<table>
<thead>
<tr>
<th>Cows</th>
<th>Heifers</th>
</tr>
</thead>
<tbody>
<tr>
<td>Herds that reported at least some pregnancy testing (N)</td>
<td>67</td>
</tr>
<tr>
<td>Median herd size (IQR)</td>
<td>200 (157 to 294)</td>
</tr>
<tr>
<td>Herds that reported pregnancy testing only a portion of the herd (N)</td>
<td>10</td>
</tr>
<tr>
<td>Herds that reported complete pregnancy testing (N)</td>
<td>57</td>
</tr>
<tr>
<td>Median herd size (IQR)</td>
<td>200 (159 to 300)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Risk of non-pregnancy</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Median (IQR)</td>
<td>6.5% (5.7% to 7.7%)</td>
</tr>
<tr>
<td>5th and 95th percentile</td>
<td>4.3%, 10.2%</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Potential risk factors for non-pregnancy other than exposure to Cfv and T. foetus (stratified by cows vs heifers) (n = 57)</th>
<th>RR (95% CI), P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Exposure to communal grazing (yes/no)</td>
<td>0.81 (0.52 to 1.27), P = 0.36</td>
</tr>
<tr>
<td>Herd size &gt; 300 cows (yes/no)</td>
<td>0.82 (0.47 to 1.43), P = 0.48</td>
</tr>
<tr>
<td>Time of bull exposure (before June/June or later)</td>
<td>1.82 (0.88 to 3.75), P = 0.11</td>
</tr>
<tr>
<td>Calving started before March 2014 (yes/no)</td>
<td>1.38 (0.72 to 2.62), P = 0.33</td>
</tr>
<tr>
<td>Calving season &gt; 4 cycles or 84 d (yes/no)</td>
<td>1.13 (0.63 to 2.03), P = 0.67</td>
</tr>
<tr>
<td>Sold purebred cattle (yes/no)</td>
<td>2.17 (0.89 to 5.32), P = 0.09</td>
</tr>
</tbody>
</table>

IQR — Interquartile range; RR — Risk ratio; CI — Confidence interval.
specificity \((Cfv)\) as \((6)\). Specificity was reported as \(T. \text{foetus} (CI), 80.6 \text{ to } 89.2; 222/260\) for \(S. \text{foetus}\), sensitivity \((Cfv)\) as \(85.4\% \text{ (95\% confidence interval (CI): 83.5 to 88.6; 255/300) for } Cfv (5) \text{ and as } 100\% \text{ (95\% CI: 98.9 to 100; 337/337) for } T. \text{foetus}\). Sensitivity was reported as \(85.0\% \text{ (95\% CI: 80.5 to 88.6; 255/300) for } Cfv (5) \text{ and as } 100\% \text{ (95\% CI: 98.9 to 100; 337/337) for } T. \text{foetus} (4)\). The apparent prevalence and sensitivity and specificity estimates reported from original validation studies \((4–6)\) using publicly available software \((13)\). Results were reported for 20 000 iterations. The parameters for the beta distribution for the individual animal results were as follows: \(\alpha = x + 1 \text{ and } \beta = n - x + 1\), where \(x\) successes out of \(n\) individuals examined in the original test validation study.

For the herds that had positive test results, the probability of getting the observed number of positive tests if the herd was free of infection was determined using publicly available software \((13)\). The calculations considered the limitations of the sensitivity and specificity of the test, as well as the total number of bulls tested. Herd level sensitivity and specificity were calculated based on the median and 95th percentile for the number of bulls tested per herd. To estimate herd level sensitivity and specificity, 2 positive reactors for \(Cfv\) and 1 positive reactor for \(T. \text{foetus}\) were required, assuming a design prevalence of 0.1 and binomial distribution \((13)\). These herd level sensitivities and specificities were then used to estimate the range for true herd prevalence \((13)\).

Finally, the distribution of expected positive and negative predictive values (post-test probabilities of infection with a positive test or freedom from infection with a negative test) were simulated for a range of potential pre-test probabilities of infection using MCMC software \((\text{ModelRisk Industrial Version 5.2; Vose Software, Sint-Amandsberg, Belgium})\) based on reported sensitivity and specificity estimates \((n = 10 000 \text{ iterations}) (4–6,14)\).

### Table III. Distribution of simulation results for true prevalence \(Cfv\) and \(T. \text{foetus}\) summarizing individual bull PCR results on direct preputial samples \((n = 735 \text{ bulls, 78 herds})\)

<table>
<thead>
<tr>
<th></th>
<th>Median</th>
<th>2.5% percentile</th>
<th>97.5% percentile</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Cfv individual bulls</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>True prevalence</td>
<td>&lt; 0.01%</td>
<td>&lt; 0.01%</td>
<td>&lt; 0.01%</td>
</tr>
<tr>
<td><strong>T. foetus individual bulls</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>True prevalence</td>
<td>&lt; 0.01%</td>
<td>&lt; 0.01%</td>
<td>0.38%</td>
</tr>
</tbody>
</table>

* Simulations \((n = 20 000)\) assume a binomial distribution for sensitivity \((\text{Cfv } \alpha = 223, \beta = 39; T. \text{foetus } \alpha = 110, \beta = 13)\) and specificity \((\text{Cfv } \alpha = 256, \beta = 46; T. \text{foetus } \alpha = 338, \beta = 1)\).

### Results

#### Study population

A group of 105 producers who were recruited to the surveillance network and completed the first survey were invited to participate. A total of 78 producers provided samples from 735 bulls between March 27, 2015 and July 16, 2015. Characteristics of the sample population are provided in Table I. The median number of bulls tested per herd was 7 \([\text{interquartile range (IQR): 5 to 12, 95th percentile range (24)}\] and the number of bulls tested per herd ranged from 1 to 37.

#### Pregnancy testing results in fall 2014 and associated herd management factors

Of the 78 producers who submitted samples for testing, 72 \(92\%\) also responded to either a paper or electronic survey about outcomes of pregnancy testing in fall 2014 \((\text{Table II})\). Complete pregnancy test results were available for 57 herds. Most producers who only checked a portion of their herd reported that they did not check cows that were going to be culled for age, temperament, or health reasons. Heifers were more likely to be open than cows \((\text{RR: 1.72, 95\% CI: 1.19 to 2.50, } P = 0.004)\) and stratification for heifers and cows was included in all subsequent models. None of the other herd management risk factors examined was significantly associated with the risk of non-pregnancy \((\text{Table II})\).

#### Laboratory results in spring 2015

Ten bulls from 3 herds \((\text{Herd A, B, and C})\) tested positive for \(Cfv\) or suspect test positive for \(T. \text{foetus}\). Eight bulls from 2 herds \((5 \text{ bulls from Herd A and 3 bulls from Herd B})\) tested positive at least once for \(Cfv\). One bull from Herd A and 1 from Herd C was suspect test positive for \(T. \text{foetus}\). No bulls tested positive for \(T. \text{foetus}\).

In Herd A, 1 sample was reported as \(T. \text{foetus}\) suspect from the 37 samples submitted on May 7, 2015. This bull was retested and was again suspect on May 29, 2015. One bull was also \(Cfv\) positive on the first submission of samples from 37 bulls on May 7, 2015. Four additional bulls were test positives by both labs from the 37 samples in the repeat submission from the same herd on May 29, 2015. The age of the \(T. \text{foetus}\) suspect was not available, but 3 of the \(Cfv\)-positive bulls were 2 y old and 2 were 3 y old. It was reported that 29.4% of the cows and 27.1% of the heifers in Herd A were not pregnant in the fall of 2014. The herd owner reported that 36 of 37 bulls tested had not been exposed to communal grazing. This question was not answered for the final bull, although this bull did not have either a positive or suspect test result. All sampled bulls from this herd were semen tested in the spring of 2015.

In Herd B, 1 of 23 bulls initially examined was test positive for \(Cfv\) on June 11, 2015. This bull and 1 other also tested positive when the same samples were submitted to a commercial laboratory \((\text{PDS})\). From a second submission of 11 bulls, a third bull was \(Cfv\) test positive on June 29, 2015. Of the 3 bulls that tested positive at least once for \(Cfv\), 1 bull was 2 y old and 1 bull was 4 y old; the age of the final bull was not reported. In Herd B, an open rate of 8.9% was reported for cows and 13.6% for heifers in the fall of 2014. One bull had been exposed to a community pasture, but tested negative for both \(Cfv\) and \(T. \text{foetus}\). All sampled bulls from this herd were semen tested in the spring of 2015.

In Herd C, 1 bull of 11 was identified as a suspect positive for \(T. \text{foetus}\) on April 20, 2015. The age of the positive bull was not reported. Herd C did not report pregnancy test data for the fall of 2014, but had indicated on the initial submission that there were no problems with pregnancy rates in the fall of 2014. None of the bulls had been exposed to a community pasture and all sampled bulls from this herd were semen tested in the spring of 2015.
Individual bull and herd-level prevalence

The crude apparent individual bull prevalence for *Cfv* was 1.1% (95% CI: 0.5% to 2.1%; 8/735) and apparent herd-level prevalence for *Cfv* was 2.6% (95% CI: 0.3% to 9.0%; 2/78). The crude apparent bull prevalence for *T. foetus* was < 0.001% (95% CI: 0.0% to 0.5%; 0/735) and apparent herd-level prevalence for *T. foetus* was < 0.001% (95% CI: 0.0% to 4.6%; 0/78).

The estimated true prevalence of both *Cfv* and *T. foetus* for the individual bull test results are summarized in Table I, taking into account previously reported clinical sensitivities and specificities of the respective tests for these laboratories and the uncertainty around these estimates associated with sample sizes.

Given the reported specificity of the *Cfv* test, the probability of getting 5 or more false positives from 37 samples collected from Herd A was 0.67. Given the specificity of the *Cfv* test, the probability of getting 3 or more false positive results from 22 samples collected from Herd B was 0.66.

The estimated herd-level sensitivity for *Cfv* when testing 7 bulls (median number tested) and requiring 2 positive reactors was 0.48 and the specificity was 0.72. The estimated herd-level sensitivity for *Cfv* when testing 24 bulls (95th percentile of bulls tested) and requiring 2 positive reactors was 0.98 and the specificity was 0.11. The estimated true herd-level prevalence for *Cfv* was 0.0% (95% CI: 0.0% to 7.3%), assuming a median of 7 bulls tested per herd.

In contrast, the estimated herd-level sensitivities for *T. foetus* when testing 7 and then 24 bulls and requiring 1 reactor were 0.48 and 0.90 and the specificities were 0.999 and 0.999. Based on the median number of 7 bulls tested per herd, the estimated true herd-level prevalence for *T. foetus* was 0.0% (95% CI: 0.0% to 9.8%).

Given the previously reported specificity for the *Cfv* qPCR, the expected number of false positive results for 735 samples was 112 (95% CI: 83 to 143). However, only 8 bulls were reported as test positive for *Cfv* in this study. If the previously reported specificity was valid for this group of samples, the probability of getting 8 or fewer false positives was < 0.0001.

Association between laboratory results and the risk of non-pregnancy in fall 2014

For the 57 herds in which pregnancy test results were reported for all cows or heifers, the association between *Cfv* test results for the bulls and the risk of non-pregnancy was modified by the difference between cows and heifers (P < 0.001). Cows from herds where at least 1 bull was test positive for *Cfv* were 2.35 times more likely (95% CI: 1.01 to 5.48, \( P = 0.047 \)) to not be pregnant than cows from herds where no positive bulls were identified. However, there was no significant increase in the risk of non-pregnancy for heifers (RR: 1.41, 95% CI: 0.77 to 2.58, \( P = 0.27 \)) in herds where at least 1 bull was test positive for *Cfv*. There were no other significant predictors or important confounders in the final model.

Also related to the significant interaction (\( P < 0.001 \)) between age group and positive *Cfv* tests for any of the bulls in the final model was whether there was a difference between cows and heifers in herds with and without *Cfv* positive bulls. Heifers were significantly more likely to be non-pregnant than cows in herds with no bulls that were *Cfv* positive (RR: 1.78, 95% CI: 1.20 to 2.65, \( P = 0.004 \)). In herds with *Cfv*-positive bulls, however, there was no difference between cows and heifers (RR: 1.41, 95% CI: 0.77 to 2.58, \( P = 0.27 \)).

The association between *T. foetus* status and risk of non-pregnancy was not considered because test results were suspect only and not confirmed as positive in any herd. Follow-up data on pregnancy testing outcomes from the fall of 2015 were not available for the 3 herds with either *Cfv* test positive or *T. foetus* suspect bulls in the spring of 2015.

Estimation of positive and negative predictive values for different pre-test probabilities of disease

Using previously published estimates of clinical sensitivity and specificity (4–6), the post-test probability of infection or positive predictive value of the direct qPCR for *Cfv* was < 90% when the pre-test probability of infection was ≤ 60% (Figure 1A) and < 40% when...
the pre-test probability of infection was ≤ 10%. When the pre-test probability of infection with Cfe was > 80%, the positive predictive value of the test was > 95% and for a pre-test probability > 95%, the positive predictive value was > 99% (Figure 1A). In contrast, when the pre-test probability of infection with T. foetus was ≥ 5%, the positive predictive value of the test was > 95% and for a pre-test probability > 20%, the positive predictive value was > 99% (Figure 2A).

The negative predictive values for a single direct qPCR test for the Cfe was > 95% when the pretest probability of disease was < 20% and > 99% when the pretest probability of disease was < 5% (Figure 1B). Similarly, the negative predictive values for a single direct qPCR test for the T. foetus was > 95% when the pretest probability of disease was < 30% and > 99% when the pretest probability of disease was < 5% (Figure 2B).

Discussion

The results of this study suggest that infections with Cfe and T. foetus were rare among breeding bulls from a sentinel cohort of cow-calf herds in western Canada. No bulls were positive for T. foetus infection and there were only 2 suspects. Given the relatively small number of herds sampled in this cohort and the imperfect nature of the tests, however, and based on the upper bound of the 95% CI, the true individual bull prevalence of T. foetus was less than 0.3% and the herd-level prevalence was less than 9.4%. The true prevalence adjusts for validity of the chosen test and is consistent with recent reports from Colorado and Nebraska, areas of the US with comparable management (8). While the sensitivity of the T. foetus test reported here was based on direct PCR of preputial samples collected into PBS (6), the specificity used in calculations may not be completely valid for PBS as it was based on samples collected into commercial sampling media (4).

The Cfe results were more difficult to interpret. While true individual bull and herd-level prevalence of Cfe was estimated to be less than 0.001 based on the previously published sensitivity and specificity of the test, 2 of 78 herds had a total of 8 test-positive bulls. There was also a decreased probability that cows from the herds with an infected bull were pregnant in the breeding season immediately before the test date. This significant association with pregnancy outcomes suggests that the test results might not be false positives as suggested by calculations of true prevalence based on reported test specificity and sensitivity.

The association between qPCR results from direct preputial samples and pregnancy outcomes is in direct contrast to the results of a previous study from New Zealand (15). The New Zealand study identified 29% of 222 tested bulls from 30 beef breeding farms as positive using a similar testing strategy. The authors attributed the lack of a significant association between test results and pregnancy outcomes to a lack of primer specificity. The authors cited previous reports from other regions noting the presence of the parA gene on mobile genetic elements and isolation of Campylobacter hyointestinalis from a bull that had tested positive through direct PCR (16-18). They also note that Cfe had not been successfully cultured in New Zealand since 1993 (15).

The present study differs from the New Zealand study (15) in that Cfe has been cultured from herd outbreaks in western Canada (1) and cultures of clinical isolates have been further verified using whole genome sequencing (Waldner, unpublished data). One of the limitations of the present study compared to the earlier New Zealand study was that there was not enough detail about herd management to directly link bull test results to pregnancy results on individual breeding pastures.

While a study of virgin bulls previously reported that the specificity of the qPCR on direct preputial samples was 85% (5), the current results suggest that the published specificity for adult bulls in western Canada is probably underestimated. There were 8 test-positive Cfe results in the present study. Even if we assume that they were all false positives, the probability of having observed 8 or fewer test-positive results was less than 0.0001. The number of observed test positives is far too low to be consistent with the previously reported specificity.

Other evidence that the specificity of the primers used in this qPCR is probably higher for mature bulls in western Canada than previously reported comes from the regional commercial laboratory. Prairie Diagnostic Services reported only 9 positive samples from 468 tested from June 2, 2014 to May 12, 2015 (19). These observations are consistent with previous reports that the effectiveness of
PCR primers for differentiating between subspecies of C. fetus varies among regions (20).

As there were no herds that were test positive for T. foetus and only 2 herds that were test positive for Cfo, there wasn’t sufficient power in this sample to look at risk factors for positive herd status. A recent survey of 863 participants in Wyoming identified grazing on public allotments and commingling with other herds as risk factors for T. foetus infection (21). In the present study, community pasture exposure was not reported for the test-positive bulls, although 1 herd had at least 1 other bull that had been exposed to communal grazing. There was also no association between community pasture exposure and an increased risk of non-pregnancy in this sentinel cohort.

In another previous study of factors associated with pregnancy outcomes in western Canada, community pasture exposure was associated with an increased risk of non-pregnancy, but only for herds that had not been vaccinated for bovine viral diarrhea virus (BVDV) and infectious bovine rhinotracheitis (IBR). That suggested a higher risk of exposure to infectious disease associated with mixing with other herds in community pastures (22). In the present study, the low prevalence of infection and lack of association between community pasture exposure and pregnancy could be in part attributed to the relatively low frequency of community pasture exposure. Exposure to communal grazing or potential for exposure to bulls or cows from other herds was reported for 10% of bulls from 28% of herds.

Increasing age has also been associated with T. foetus infection (7). In the present study, age was not reported for the 2 bulls with suspect T. foetus test results, although the bulls that were test positive for Cfo ranged in age from 2 to 4 y.

The final objective of this study was to compare the predictive values of positive and negative test results for both T. foetus and Cfo when screening bulls in the absence of a recognized problem with reproductive performance (low pre-test probability) and when examining bulls as part of the diagnosis of poor pregnancy rates (high pre-test probability). The probability that a positive test result from the direct PCR test indicates T. foetus infection is greater than 90% for pre-test probabilities as low as 5%. The positive predictive value drops off quickly, however, as the prevalence of infection in the target population dips below 1%. Similarly, the negative predictive value is greater than 90% until the pre-test probability of infection exceeds 50%. Taken together, the direct PCR T. foetus for preputial samples collected directly into PBS can be used for screening low- and moderate-risk bulls with an acceptable probability of false-positive results. However, the probability of false positive becomes very high for very low risk situations such as screening virgin yearling bulls with no history of exposure to an infected herd.

The direct PCR test for T. foetus also has high post-test probability for negative results, but due to the imperfect sensitivity, the risk of false negatives from a single test is very high when clinical suspicion of T. foetus is above 50%. The sensitivity of the test based on a single sample is less than 100% even when PCR is used with commercial media (4). Trichomonas foetus is not always recovered from infected bulls during sampling due to the small number of organisms, inconsistent presence, and uneven distribution in the prepulse (7).

This emphasizes the need for 3 repeated tests from at least weekly intervals to rule out infection when clinical suspicion is moderate or high at the time of testing. The 3-sample strategy was recommended in a recent review of T. foetus prevention and control regardless of whether culture or PCR was used for testing (8). Based on the simulation results from this study, a single test for T. foetus is not appropriate if the pre-test probability of infection is higher than 1%, based on available evidence.

As we are limited to the current reported estimates of specificity of the Cfo test, the direct PCR for Cfo should not be recommended for routine screening of moderate- and low-risk bulls at this time. The positive predictive value appears to be less than 85% when the pre-test probability of disease is 50% or lower. The costs of false positives that could result from applying the test to low- and very low-risk bulls include the costs of testing, premature culling of valuable bulls, and other potential control measures such as herd vaccination programs. Virgin bulls were excluded from the present study due to the potential for false positives. If positive test results could be confirmed by retesting the bull with culture, the costs of screening moderate-risk bulls might be acceptable. However, most bulls in western Canada are located in areas where the transport time to the lab is greater than 24 h, which compromises the sensitivity of culture (2). As a result, very few herds have access to confirmatory culture with transport times short enough to ensure very good sensitivity, regardless of the use of transport media (9).

The direct PCR test for Cfo could potentially be used as a diagnostic test when investigating high-risk bulls from herds with outbreaks of poor reproductive performance consistent with bovine genital campylobacteriosis. To maximize the pre-test probability and resulting interpretation of the test, however, the test is best used after ruling out other common causes of reproductive failure. Because of the limited negative predictive value in high-risk situations, 3 sequential tests have been recommended to provide strong evidence that a bull from an outbreak situation is free of infection and to definitively rule out Cfo as the cause of an outbreak (5,10).

The present study reported the results for qPCR applied to preputial scrapings collected directly into PBS. This method has previously been suggested as an opportunity to decrease sampling costs and improve ease of sample handling especially in the winter when there is a high risk of samples freezing in transport (5,6,10). The regional laboratory recommends that T. foetus samples should be collected into commercial culture media (In Pouch TF; Biomed Diagnostics) and be incubated at 35°C for 48 h before shipping warm to the laboratory. Use of commercial culture media followed by incubation increases the time and costs associated with sample collection compared to that described in the current study (23). However, the sensitivity of the qPCR for T. foetus for a single preputial sample collected into commercial culture media pouches is higher than when collecting directly into PBS, as was done in the present study (6). While this difference is important for a single sample, when 3 samples are collected as recommended, the difference in sensitivity between PBS and the commercial pouches is no longer significant (6).

Additional work is required to identify a more specific and practical field test to screen bulls for Cfo. Given the justifiable reluctance of many herd owners to handle their bulls 3 times in succession, improvements to the recommended testing strategy for T. foetus would also increase opportunities for disease control. As testing and culling are considered the primary means of managing both T. foetus infection and Cfo (2), it is important to understand the limitations.
and carefully consider how to make the best possible use of the currently available tests while minimizing the risks and costs associated with false positive and negative results.

References


Efficacy of an accelerated hydrogen peroxide disinfectant to inactivate porcine epidemic diarrhea virus in swine feces on metal surfaces

Derald J. Holtkamp, Jacqueline Myers, Paul R. Thomas, Locke A. Karriker, Alejandro Ramirez, Jianqiang Zhang, Chong Wang

Abstract

In May of 2013, porcine epidemic diarrhea virus (PEDV) was detected in swine for the first time in North America. It spread rapidly, in part due to contaminated livestock trailers. The objective of this study was to test the efficacy of an accelerated hydrogen peroxide disinfectant for inactivating PEDV in the presence of feces on metal surfaces, such as those found in livestock trailers. Three-week-old barrows were inoculated intragastrically with 5 mL of PEDV-negative feces for the negative control, 5 mL of untreated PEDV-positive feces for the positive control, and 5 mL or 10 mL of PEDV-positive feces that was subjected to treatment with a 1:16 or 1:32 concentrations of accelerated hydrogen peroxide disinfectant for a contact time of 30 min at 20°C. These pigs served as a bioassay to determine the infectivity of virus following treatment. Rectal swabs collected from the inoculated pigs on days 3 and 7 post-inoculation were tested by using PEDV-specific real-time reverse transcription polymerase chain reaction and the proportion of pigs in each group that became infected with PEDV was assessed. None of the pigs used for the bioassay in the 4 treatment groups and the negative control group became infected with PEDV, which was significantly different from the positive control group ($P < 0.05$) in which all pigs were infected. The results suggest that the application of the accelerated hydrogen peroxide under these conditions was sufficient to inactivate the virus in feces found on metal surfaces.

Résumé

En mai 2013, pour la première fois en Amérique du Nord, le virus de la diarrhée épidémique porcine (VDEP) fut détecté chez le porc. Il se répandit rapidement, en partie à cause des remorques pour animaux contaminées. L’objectif de la présente étude était de tester l’efficacité d’un désinfectant à base de peroxyde d’hydrogène accéléré pour inacter le VDEP en présence de fèces sur des surfaces métalliques, telles que celles retrouvées dans les remorques pour animaux. Des mâles castrés âgés de 3 semaines ont été inoculés par voie intra-gastrique avec 5 mL de fèces VDEP-négatives pour les témoins négatifs, 5 mL de fèces VDEP-positives non traitées pour les témoins positifs, et 5 mL ou 10 mL de fèces VDEP-positives soumises à un traitement au désinfectant à base de peroxyde d’hydrogène accéléré à une concentration de 1:16 ou 1:32 avec un temps de contact de 30 min à 20 °C. Les porcs ont servi de bioassai afin de déterminer l’infectivité du virus suite au traitement. Des écouvillons rectaux prélevés des porcs inoculés aux jours 3 et 7 post-inoculation ont été testés par réaction d’amplification en chaîne en temps réel utilisant la transcriptase réverse et spécifique au VDEP, et la proportion de porcs devenus infectés par le VDEP dans chaque groupe fut déterminée. Aucun des porcs utilisés pour le bioassai dans les quatre groupes de traitement ainsi que dans le groupe témoin négatif ne devint infecté par le VDEP ce qui était significativement différent des animaux du groupe témoin positif ($P < 0.05$) qui devinrent tous positifs. Ces résultats suggèrent que l’application du peroxyde d’hydrogène accéléré dans les conditions testées était suffisante pour inacter le virus présent dans les fèces sur les surfaces métalliques.

Introduction

Porcine epidemic diarrhea (PED) was first described in England in 1971 in growing pigs (1), and the causative agent, porcine epidemic diarrhea virus (PEDV), was identified in 1978 (2,3). In May of 2013, PEDV was identified in swine for the first time in the United States and North America. The virus caused severe diarrhea in sows and piglets, with high mortality in neonatal piglets across a wide geographical area of the United States (4). Although the original route of entry remains unknown, contaminated livestock trailers, especially those that haul pigs to harvest facilities, represent a significant risk for movement of the virus among herds (5).

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Overnight drying and several disinfectants have been shown to effectively inactivate PRRSV after washing. Disinfectants reported to reduce the risk of PRRSV transmission associated with contaminated trailers at temperatures above 0°C include a phenolic compound (Tek-Trol; Biotek Industries, Atlanta, Georgia, USA) in combination with drying (6), 2 quaternary ammonium and glutaraldehyde combinations [Synergize; Preserve International, Atlanta, Georgia, USA (7,10) and Aseptol 2000; SEC Repro, Quebec (9)], and an accelerated hydrogen peroxide (AHP) disinfectant (Accel; Virox Technologies, Oakville, Ontario) (10). Consequently, the industry standard trailer sanitation and decontamination today consists of washing, disinfection, and drying either naturally or with a TADD system.

As the industry standard, a complete wash, disinfect, and dry is always the preferred option. The industry standard, however, takes time and requires specialized facilities. Unfortunately, there are not enough of these facilities in North America to serve the large volume of livestock trailers transporting pigs across the country every day. As an alternative to doing nothing, sanitation and decontamination procedures that involve scraping to remove as much organic material as possible followed by disinfection, heating, drying, or some combination of these without a thorough washing, if demonstrated to effectively inactivate PEDV, could be used when a complete wash, dry, and disinfection is not possible. In a previous study using PEDV-positive feces contaminated aluminum coupons as a model of full-sized livestock trailers, heating to 71°C for 10 min or allowing them to sit for 7 d at 20°C was sufficient to prevent transmission of PEDV present in feces as determined by a bioassay (11).

Accel is an AHP disinfectant registered as a disinfectant cleaner that is virucidal at dilution rates of 1:16 to 1:64, in the presence of 200 ppm hard water, 5% serum load, and a 5 min contact time. The active ingredient is hydrogen peroxide, which is an oxidizing agent. It produces a long-lasting foam when delivered through a foaming tip and contains food-grade anionic and non-ionic surfactants, which act with hydrogen peroxide to increase microbicidal activity and may make it a candidate to work in the presence of some organic matter. The aim of this study was to test the efficacy of an AHP disinfectant for inactivating PEDV in the presence of feces on metal surfaces such as those found in livestock trailers.

### Materials and methods

For this study, 15.24 cm × 15.24 cm aluminum trays with 2.54 cm high sides and a material thickness of 0.32 cm were used as “coupons” to represent the surface of livestock trailers used to haul pigs. The coupons were used in a previous study to evaluate the efficacy of different combinations of time and temperature to inactivate PEDV (11). To simulate the runoff that occurs in full-sized livestock trailers as the AHP disinfectant is transformed from foam to a liquid, the coupons were modified for this study by drilling 4 holes in each coupon that were 8 mm in diameter at the junction of the bottom of the coupon and 1 side. The experimental unit was a single contaminated coupon that was matched to a single 3-week-old pig intragastrically inoculated with the contents of the coupon, as a bioassay to determine if the treatment applied to the contaminated coupon effectively inactivated PEDV.

### Table I. Description of study groups

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>Description of contamination and treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Negative control</td>
<td>5 mL PEDV-negative feces, no treatment</td>
</tr>
<tr>
<td>Positive control</td>
<td>5 mL PEDV-positive feces, no treatment</td>
</tr>
<tr>
<td>5 mL:1:16</td>
<td>5 mL PEDV-positive feces, 1:16 concentration of AHP disinfectant&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>10 mL:1:16</td>
<td>10 mL PEDV-positive feces, 1:16 concentration of AHP disinfectant&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>5 mL:1:32</td>
<td>5 mL PEDV-positive feces, 1:32 concentration of AHP disinfectant&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>10 mL:1:32</td>
<td>10 mL PEDV-positive feces, 1:32 concentration of AHP disinfectant&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Transmission control</td>
<td>1 of 4 pigs in the group was gavaged with 5 mL PEDV-positive feces; 3 of 4 were gavaged with 5 mL PEDV-negative feces, no treatment</td>
</tr>
</tbody>
</table>

<sup>a</sup>With 30 min of contact time at 20°C (room temperature).

PEDV — porcine epidemic diarrhea virus.

The null hypothesis for the study was that there was no difference between the positive control group and the treatment groups in the proportion of pigs infected by the inoculum collected from the coupons as measured by the proportion of pigs that were positive for PEDV. The PEDV status of the pigs was assessed by testing rectal swabs collected at 3 and 7 d post-inoculation for PEDV by a nucleocapsid (N) gene-based quantitative real-time reverse transcription polymerase chain reaction (real-time RT-PCR) at the Iowa State University Veterinary Diagnostic Laboratory (ISU VDL). The primers and probe of the PEDV real-time RT-PCR have been described previously (5,12,13). Each PCR was set up in a 25 μL total reaction using an RT-PCR kit (Path-ID Multiplex One-Step RT-PCR Kit; Thermo Fisher Scientific, Waltham, Massachusetts, USA): 12.5 μL of 2 × multiplex RT-PCR buffer, 2.5 μL of 20 × multiplex enzyme, 0.5 μL of forward primer at 20 μM, 0.5 μL of reverse primer at 20 μM, 0.12 μL of probe at 25 μM, 3.88 μL of nuclease-free water, and 5 μL of nucleic acid extract. Amplification reactions were done using a real-time thermal cycler (ABI 7500 Fast instrument; Thermo Fisher Scientific) with the following conditions: 1 cycle of 48°C for 10 min, 1 cycle of 95°C for 10 min, and 45 cycles of 95°C for 15 s and 60°C for 45 s.

Personnel performing treatments, necropsies, and collecting samples were not blinded to the treatments. Blinding was not possible because a specific order was followed for all procedures starting with the negative control and ending with the positive control to minimize the risk of transmitting virus between treatment groups. Laboratory personnel who performed the PCR testing were blinded to treatment status of the pigs from which the samples were collected.

### Study groups

Pigs (n = 28) were divided into 7 groups. Four treatment groups (n = 4, per group) representing combinations of fecal contamination (5 mL or 10 mL) and disinfectant concentration (1:16 or 1:32) were
evaluated. In addition, a positive control group \((n = 4)\) and negative control group \((n = 4)\) were included without sham disinfection. The PEDV-negative feces were used to contaminate coupons in the negative control group. The PEDV-positive feces were used to contaminate coupons in the positive control group. The AHP disinfectant was applied as a foam. A good candidate for sham disinfection, to simulate the rinsing and diluting effects, would be a non-disinfecting foam similar to that produced with the AHP disinfectant; however, no such foam could be identified. Because any liquid used for a sham disinfection would have very different rinsing and dilution effects compared to the foam produced by the AHP disinfectant no sham disinfection was done. A transmission control group \((n = 4)\) was included to validate that the animal housing and handling protocols used for the bioassay did not result in transmission of virus from one pig to another within the same treatment group. The study groups are summarized in Table I.

**Contamination and disinfection procedures**

The PEDV-positive feces were obtained from a separate experiment in which 3-week-old pigs were inoculated with PEDV isolate US/Iowa/18984/2013 (13). The feces were collected from the pigs at 7 d post-inoculation. The feces from individual pigs were placed on ice until they could be frozen at \(-80^\circ C\) approximately 1 h later. On the day of the challenge, day 0, these samples were thawed and pooled into a single fecal homogenate to assure that the amount of virus and composition of the feces were uniform for each replicate. Samples from each replicate were tested at the ISU VDL by using real-time polymerase chain reaction (RT-PCR) and were positive for PEDV. The quantitative genomic copies/mL ranged from \(10^{9.61}\) to \(10^{10.17}\) genomic copies/mL across all replicates. The PEDV-negative feces were collected from the negative control pigs in the same previous study. Negative feces were frozen at \(-80^\circ C\), stored, thawed on day 0 and homogenized into a single pool at that time. These fecal samples were confirmed PEDV-negative by using RT-PCR.

Prior to treatment, 5 mL (positive control, negative control, 5 mL-1:16, 5 mL-1:32, transmission control) or 10 mL (10 mL-1:16, 10 mL-1:32) of feces were applied to the aluminum coupons (Figure 1). The 5 and 10 mL of feces were chosen to represent the amount of organic matter that remains in a livestock trailer after the feces and bedding have been manually removed with a scraper. Feces that were positive for PEDV was applied to the coupons in the following groups: 5 mL-1:16, 10 mL-1:16, 5 mL-1:32, 10 mL-1:32, positive control, and 1 of the 4 pigs in the transmission control group. Feces that were negative for PEDV were applied to the coupons in the negative control study group and 3 of the 4 pigs in the transmission control group. The feces were spread in a thin \((\leq 2\, \text{mm})\), even layer using a disposable spreader. The disposable spreader was a hard plastic spreader sold in hardware stores to spread adhesive on floors. A separate, new spreader was used for each coupon to avoid potential cross-contamination between replicates. Following application of feces, the coupons were individually swabbed using a commercial swab and transport system (StarSwab II; Starplex Scientific, Etobicoke, Ontario) that were submitted to the ISU VDL to be tested for the presence of viral RNA by RT-PCR.

A 4.25% concentrate of AHP was diluted with tap water from a municipal water source at a dilution rate of 1:16 for treatment groups 5 mL-1:16 and 10 mL-1:16 and at a dilution rate of 1:32 for treatment groups 5 mL-1:32 and 10 mL-1:32. The negative control, positive control, and transmission control groups were not sham disinfected. The AHP disinfectant was applied to all replicates \((n = 4)\) of a treatment group simultaneously. Four was the minimum number of replicates required to demonstrate statistical significance \((P < 0.05)\) for the bioassay between 0 of 4 and 4 of 4 pigs infected. A liquid volume of approximately 30 mL of diluted AHP was applied with a 5.7 L pump-up foamer (model #A8020A; Ogena Solutions, LLC, Stoney Creek, Ontario; Figure 2). Given the area of the coupons used in this study, it was calculated that 30 mL of diluted disinfectant was proportionally equivalent to the 189 L applied during a 10 min application to a typical full sized 15.8-meter double-decked livestock trailer using a proportioning foamer that attaches to the end of a hose with a flow rate of 18.9 L/min. With a series of timed applications prior to the start of the trial it was determined that 3 s per coupon would result in the desired liquid volume of 30 mL of diluted AHP at both concentrations.

The contact time with the AHP disinfectant for treatment groups 5 mL-1:16, 10 mL-1:16, 5 mL-1:32, and 10 mL-1:32 was 30 min. A minimum 30-minute contact time is attainable under nearly all circumstances encountered in the transport of swine. Contamination of

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**Figure 1.** Application of feces to the aluminum coupons. Feces were applied to the coupons using a disposable flat spreader to produce a thin even layer on the floor of the coupon.

**Figure 2.** Application of accelerated hydrogen peroxide disinfectant to the aluminum coupons. Disinfectant was applied as a foam using a commercially available 5.7 L pump-up foamer.
the coupons, application of the AHP disinfectant and the 30-minute contact time occurred at 20°C. For the positive control, negative control, and transmission control groups, contamination occurred at 20°C, and the coupons were held at that temperature for 30 min before being collected for the bioassay.

Thirty minutes after contamination (positive control, negative control, and transmission control) or application of the AHP disinfectant (5 mL-1:16, 10 mL-1:16, 5 mL-1:32, and 10 mL-1:32), the coupons were swabbed using the same commercial swab and transport system used to swab the coupons immediately after contamination. The swabs were submitted to the ISU VDL to be tested for the presence of viral RNA by RT-PCR. The coupons were then tilted so that the remaining material in the coupon flowed away from the holes, and 10 mL of sterile 0.9% sodium chloride saline solution (Hospira, Lake Forest, Illinois, USA) was added to each coupon. A new toothbrush, one per coupon, was used to suspend the remaining feces and disinfectant for ease of re-collection. The liquid mixture of feces, remaining AHP disinfectant and saline was aspirated using a 20 mL syringe (Figure 3). The syringe was capped and labeled with the identification number of the single pig that was to receive the mixture. Nitrile gloves (VetOne; MWI Veterinary Supply Company, Boise, Idaho, USA) were worn and changed between each coupon during collection to prevent possible cross-contamination between plates.

**Source of animals and housing for bioassay**

The experimental protocol was approved by the Iowa State University Institutional Animal Care and Use Committee (Log Number: 6-14-7812-S) and the Iowa State University Institutional Biosafety Committee (Log Number: 14-I-0022-A) prior to initiation of any experimental activity. The study was carried out in strict adherence to IACUC guidelines regarding humane use of animals. Twenty-eight, 3-week-old, clinically healthy barrows were sourced from a private commercial producer in Iowa. On day –1 of the study, 72 h after arrival, blood was collected from each pig via jugular venipuncture using a 12-mL syringe with a 38 mm 18-gauge needle (Monoject; Covidien, Mansfield, Massachusetts, USA), then transferred to an 8.5 mL plastic serum separator tube (BD Vacutainer, 8.5 mL draw; Becton, Dickinson and Company, Franklin Lakes, New Jersey, USA). The blood was centrifuged at 2100 × g for 10 min. The serum was aliquoted into two 5-mL snap cap tubes (BD Falcon polypropylene round-bottom tube; Becton, Dickinson and Company). One aliquot was frozen and stored at −80°C and the other was submitted to ISU VDL for diagnostic testing. Rectal swabs were collected using a commercially available swab and transport system (Starswabs II) and submitted to the ISU VDL for diagnostic testing. Rectal swabs were tested for PEDV (13), porcine deltacoronavirus (PDCoV) (14), and transmissible gastroenteritis virus (TGEV) (10) by virus-specific RT-PCR. Serum samples were tested for PRRSV using a commercial RT-PCR (VetMax NA; Waltham, Massachusetts, USA) and European (EU) PRRSV RT-PCR (Thermo Fisher Scientific) following the manufacturer’s instructions. Serum samples were tested for antibodies to PEDV by immunofluorescence assay (IFA) following previously described procedures (12) and antibodies for TGEV using a commercial enzyme-linked immunosorbent assay (ELISA) (SVANOVIR TGEV/PRCV-Ab differential ELISA; Svanova Biotech AB, Uppsala, Sweden).

On arrival, each pig was identified with a unique plastic ear tag (Allflex USA, Dallas, Texas, USA) and weighed. Pigs were blocked by weight and randomly assigned to 1 of 7 groups (n = 4) using computer software (RAND function in Microsoft Excel, version 2010; Microsoft Corporation, Redmond, Washington, USA). Each group was housed in a separate room in the Iowa State University Veterinary Medical Research Institute for the duration of the study. The 4 pigs within each group were housed individually in elevated tubs constructed for a previous study involving a swine bioassay (11). The tubs had solid dividers completely separating each of the 4 pigs from one another. Each divided portion of the tub had dedicated water and feed sources (Figure 4). Pigs were fed an age-appropriate corn and soybean meal based diet ad libitum that was free of medications and any ingredients of porcine origin. Feces fell through the plastic, slatted flooring of the tub into a common collection area below the pigs, where it drained into a holding container to minimize the potential for environmental contamination.

**Inoculation of pigs for bioassay**

The collected mixture of inoculum was then immediately used to inoculate the pigs for the bioassay. This was considered day 0 of

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**Figure 3. Collection of material from aluminum coupons used to inoculate pigs for the bioassay. A liquid mixture of feces, disinfectant, and saline was aspirated using a 20-mL syringe that was labeled with the pig number of the single pig to be inoculated with the contents from a single coupon.**

**Figure 4. Elevated tubs used to house pigs for duration of the study. Tub walls divided into 4 quadrants, each with dedicated water and feed. All 4 pigs in each group were housed in the same tub, 1 pig per quadrant.**
the study. Personnel performing the inoculation wore disposable coveralls (Tyvek coveralls; DuPont, Wilmington, Delaware, USA) and a respirator (N95; 3M, St. Paul, Minnesota, USA) that were changed between groups. Additionally, personnel wore arm-length disposable obstetrical sleeves (Agri-Pro Enterprises, Iowa Falls, Iowa, USA), and nitrile gloves that were changed between each pig to prevent cross-contamination. Following the inoculation of each pig and discarding of the obstetrical sleeves and gloves, the coveralls were examined for possible contamination. If any contamination was observed, the coveralls were removed, discarded, and a new pair was donned. Inoculation was performed via gastrogavage, as previously described (11) using a 14 French rubber catheter (Kendall Covidien, Mansfield, Massachusetts, USA).

Following inoculation, clinical signs, including diarrhea, were assessed daily. On days 3 and 7 post-challenge, samples of feces from each pig were collected with rectal swabs (Starswabs II) and tested for PEDV by RT-PCR. The same biosecurity procedures using coveralls, masks, gloves, and obstetrical sleeves used when pigs were

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>Pre-treatment b</th>
<th>Percentage positive or suspect for PEDV</th>
<th>Post-treatment c</th>
<th>Percentage positive or suspect for PEDV d</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Ct value and genomic copies/mL</td>
<td></td>
<td>Ct value and genomic copies/mL</td>
<td></td>
</tr>
<tr>
<td>Negative control e</td>
<td>&gt; 40 (0)</td>
<td>0% (0 of 3)</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>Positive control</td>
<td>14.2 (10^9.73)</td>
<td>100% (4 of 4)</td>
<td>36.1 (10^3.28)</td>
<td>75% (3 of 4)</td>
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<td>5 mL-1:16</td>
<td>12.7 (10^10.17)</td>
<td></td>
<td>36.4 (10^3.19)</td>
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<td></td>
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</tr>
<tr>
<td>10 mL-1:16</td>
<td>14.3 (10^9.70)</td>
<td>100% (4 of 4)</td>
<td>38.7 (10^2.52)</td>
<td>100% (4 of 4)</td>
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<td></td>
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<td>37.4 (10^2.80)</td>
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</tr>
<tr>
<td></td>
<td>13.9 (10^9.82)</td>
<td></td>
<td>35.5 (10^3.46)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>13.2 (10^10.02)</td>
<td></td>
<td>35.0 (10^3.61)</td>
<td></td>
</tr>
<tr>
<td>5 mL-1:32</td>
<td>14.3 (10^9.70)</td>
<td>100% (4 of 4)</td>
<td>37.6 (10^2.84)</td>
<td>50% (2 of 4)</td>
</tr>
<tr>
<td></td>
<td>14.1 (10^9.76)</td>
<td></td>
<td>32.2 (10^4.43)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>14.2 (10^9.73)</td>
<td></td>
<td>&gt; 40 (0)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>14.6 (10^9.61)</td>
<td></td>
<td>&gt; 40 (0)</td>
<td></td>
</tr>
<tr>
<td>10 mL-1:32</td>
<td>13.8 (10^9.85)</td>
<td>100% (4 of 4)</td>
<td>34.3 (10^3.81)</td>
<td>50% (2 of 4)</td>
</tr>
<tr>
<td></td>
<td>13.7 (10^9.88)</td>
<td></td>
<td>&gt; 40 (0)</td>
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</tr>
<tr>
<td></td>
<td>13.6 (10^9.91)</td>
<td></td>
<td>37.6 (10^2.84)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>14.2 (10^9.73)</td>
<td></td>
<td>&gt; 40 (0)</td>
<td></td>
</tr>
<tr>
<td>Transmission control</td>
<td>14.4 (10^9.67)</td>
<td>25% (1 of 4)</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td></td>
<td>&gt; 40 (0)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>&gt; 40 (0)</td>
<td></td>
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<td></td>
</tr>
</tbody>
</table>

a Treatment groups are summarized in Table I.

b Results for swabs of coupons following contamination with feces before exposure to AHP disinfectant for 30 min of contact time. Genomic copies/mL in parenthesis.

c Results for swabs of coupons following treatment with AHP disinfectant for 30 min of contact time. Negative control, positive control, and transmission control groups were not sham disinfected and no “post-treatment” swabs were collected for these groups.

d Cycle threshold (Ct), genomic copies and percentage positive or suspect reported. Positive, Ct < 35; Suspect, 35 ≤ Ct < 40; Negative, Ct ≥ 40.

e One pig in the negative control group died prior to initiation of the bioassay (day 0).

N/A — Not available.
intragastrically inoculated, were used when sampling pigs. During sampling, pigs were not removed from their individual pens to avoid cross-contamination between individuals. Bioassays were considered to be positive if the fecal samples were positive for PEDV by RT-PCR on days 3 and 7. A Ct value less than 35 was considered positive.

Following collection of rectal swabs on study day 7, all animals were humanely euthanized using penetrating captive bolt and necropsied. Gross evaluation was performed on all organ systems and any gross pathology noted. From each pig, fresh cecal and spiral colon contents, sections of fresh and 10% formalin-fixed ileum, and fresh and formalin-fixed mesenteric lymph nodes were collected. Fresh samples were immediately frozen at −80°C, and all samples were held in the event further testing might be required to confirm the results obtained on rectal swabs by RT-PCR.

Statistical analysis was done using computer software (SAS version 9.3; SAS Institute, Cary, North Carolina, USA), with P < 0.05 considered statistically significant. Pre-treatment Ct values were compared between groups using analyses of variance (ANOVA). Difference in Ct values between pre- and post-treatment were assessed for each group using linear mixed models and compared between groups using an F-test. Analysis of the bioassay results was done using Fisher’s exact test to evaluate differences in proportions of pigs positive for the bioassay between groups.

## Results

One of the 4 pigs in the negative control group died on study day −1 after the pigs were assigned to the study groups but before being inoculated on day 0. The pig was submitted to the ISU VDL for a full necropsy and diagnostic workup from which it was concluded that the cause of death was not linked to any study procedures. No other animals were removed from the study. The diagnostic results from samples taken on study day −1 confirmed that the pigs were negative for PEDV, PDCoV, PRRSV, and TGEV by RT-PCR. All of the pigs were negative for antibodies to PEDV by IFA and for antibodies to TGEV by differential ELISA.

The summary of PEDV RT-PCR results for swabs of contaminated coupons before and after treatment is presented in Table II. The Ct values and the quantitative genomic copies/mL are both reported. Swabs taken immediately after contamination (pre-treatment) from all of the coupons contaminated with PEDV-negative feces in the negative control group and the 3 coupons in the transmission control group that were designated as negative (3 of 4) tested negative for PEDV by RT-PCR. Swabs taken immediately after contamination from all of the coupons contaminated with PEDV-positive feces in the positive control group (4 of 4) and the 1 coupon in the transmission control group that was designated as positive (1 of 4) tested positive for PEDV by RT-PCR with the quantitative results ranging from 10^{9.61} to 10^{9.91} genomic copies/mL. Swabs from coupons contaminated with PEDV-positive feces in the treatment groups (5 mL-1:16, 10 mL-1:16, 5 mL-1:32, and 10 mL-1:32) collected immediately after contamination and before treatment with AHP were all positive for PEDV by RT-PCR with the quantitative results ranging from 10^{9.61} to 10^{10.17} genomic copies/mL. Pre-treatment Ct values for the groups contaminated with PEDV-positive feces (positive control, 5 mL-1:16, 10 mL-1:16, 5 mL-1:32, and 10 mL-1:32) were not significantly different (P = 0.07). Swabs from coupons contaminated with PEDV-positive feces in the treatment groups using the 1:16 concentration of AHP (5 mL-1:16, 10 mL-1:16) collected after exposure to AHP for 30 min of contact time were all either negative (Ct ≥ 40) or suspect (35 ≤ Ct < 40) for PEDV by RT-PCR. The highest concentration was 10^{6.41} genomic copies/mL. Treatment groups with the 1:32 concentration of AHP (5 mL-1:32 and 10 mL-1:32) each had one positive (Ct < 35) and one suspect swab collected after 30 min of contact time with the less concentrated AHP for 30 min and the highest concentration was 10^{4.3} genomic copies/mL. However, only half of the swabs from trays treated with the 1:32 concentration of AHP (5 mL-1:32 and 10 mL-1:32) were positive or suspect compared to 75% or 100% of the swabs in the trays treated with the higher concentration of AHP (5 mL-1:16, 10 mL-1:16). The increase in Ct values between pre- and post-treatment were significantly different from zero for all treatment groups (5 mL-1:16, 10 mL-1:16, 5 mL-1:32, and

### Table III. Summary of porcine epidemic diarrhea virus (PEDV) real-time reverse transcription polymerase chain reaction (RT-PCR) results for rectal swabs collected on days 3 and 7 post-inoculation for the swine bioassay

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>Day 3 post-inoculation: percentage positive for PEDV (positive/tested)</th>
<th>Day 7 post-inoculation: percentage positive for PEDV (positive/tested)</th>
<th>Bioassay result: percentage positive for PEDV (positive/tested)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Negative control</td>
<td>0% (0/3)</td>
<td>0% (0/3)</td>
<td>0% (0/3)</td>
</tr>
<tr>
<td>Positive control</td>
<td>100% (4/4)</td>
<td>0% (0/4)</td>
<td>100% (4/4)</td>
</tr>
<tr>
<td>5 mL-1:16</td>
<td>0% (0/4)</td>
<td>0% (0/4)</td>
<td>0% (0/4)</td>
</tr>
<tr>
<td>10 mL-1:16</td>
<td>0% (0/4)</td>
<td>0% (0/4)</td>
<td>0% (0/4)</td>
</tr>
<tr>
<td>5 mL-1:32</td>
<td>0% (0/4)</td>
<td>0% (0/4)</td>
<td>0% (0/4)</td>
</tr>
<tr>
<td>10 mL-1:32</td>
<td>0% (0/4)</td>
<td>0% (0/4)</td>
<td>0% (0/4)</td>
</tr>
<tr>
<td>Transmission control</td>
<td>25% (1/4)</td>
<td>25% (1/4)</td>
<td>25% (1/4)</td>
</tr>
</tbody>
</table>

a Treatment groups are summarized in Table I.

b Values with different letters were significantly different (P < 0.05) by Fishers exact test. The transmission control group was included to validate the animal housing and handling protocols used for the bioassay. Values for transmission control group were not compared to those of the other groups.
The summary of PEDV RT-PCR results for rectal swabs collected from pigs on days 3 and 7 post-inoculation for the swine bioassay are reported in Table III. Rectal swabs collected from all pigs in the negative control group, the 3 pigs designated as negative (3 of 4) in the transmission control group and all pigs in the 4 treatment groups (5 mL-1:16, 10 mL-1:16, 5 mL-1:32, and 10 mL-1:32) were negative for PEDV by RT-PCR on days 3 and 7 post-inoculation. Rectal swabs collected from all pigs in the positive control group and from the 1 pig designated as positive (1 of 4) in the transmission control group were all positive for PEDV by RT-PCR on days 3 and 7 post-inoculation. Rectal swabs in any group had Ct values within the suspect range (35 ≤ Ct < 40).

A Fisher’s exact test was performed to evaluate differences between groups in the proportion of pigs positive for the bioassay. Each pig was considered positive for the bioassay if the fecal samples from the pig were positive for PEDV by real-time RT-PCR on days 3 and 7. The proportion of pigs that were positive for the bioassay in the negative control and all of the treatment groups (5 mL-1:16, 10 mL-1:16, 5 mL-1:32, and 10 mL-1:32) were significantly different than the positive control (P < 0.05).

**Discussion**

An AHP disinfectant inactivated PEDV in the presence of significant feces on metal surfaces like those found in livestock trailers, at room temperature with 30 min of contact time. The PEDV was inactivated by both concentrations of the AHP disinfectant evaluated, 1:16 and 1:32, and when 5 mL and 10 mL of feces were present. Wood shavings are frequently used as bedding in livestock trailers that haul swine. Inclusion of wood shavings was considered; however, the type of wood and size of the shavings used as bedding varies considerably and some types of wood have been shown to have virucidal properties (15). Therefore, feces alone without wood shavings was used to contaminate the coupons in the current study to avoid the potentially confounding effect of the choice of shavings.

Because disinfection, natural drying, or TADD systems are more effective when applied to a clean trailer with little or no remaining organic matter, the industry standard for sanitation and decontamination of livestock trailers includes trailer washing, disinfection, and natural drying or TADD systems. When a complete wash, disinfection, and drying cannot be done, due to lack of resources or other logistical constraints, the results of this study suggest that scraping livestock trailers to remove as much organic material as possible followed by disinfection with an AHP, may be used as an alternative to doing nothing to reduce the risk of PEDV transmission associated with livestock trailers.

Limitations of current viral isolation methods in cell culture make it difficult to culture PEDV outside of an animal model. Currently swine bioassay remains the best means to determine if infectious PEDV is present in a sample. The use of a bioassay also eliminates the possible negative impact, such as cytotoxicity, of feces and disinfectant present in a sample on virus isolation outcomes in cell culture. In the current study, a 3-week-old pig bioassay model was used to assess the effectiveness of an AHP disinfectant on inactivating PEDV. Three-week-old pigs were used because PEDV does not typically cause mortality in this age of pig but they are still highly susceptible to infection. Thomas et al (12) reported that 100% of 21-day-old pigs inoculated with 10 mL of a virulent PEDV prototype isolate with titers of 560–56 TCID 50/mL were infected while 10 mL of inoculum with titers 0.56–0.0056 TCID 50/mL failed to infect the 21-day-old pigs.

The PCR results for the swabs collected from the coupons after treatment with the AHP suggest that, in most cases, the disinfectant degraded the genetic material of the virus to the extent that the amount of intact RNA was close to or below the limit of detection of the RT-PCR assay used in this study. Of the 16 replicates in the 4 treatment groups with AHP (5 mL-1:16, 10 mL-1:16, 5 mL-1:32, and 10 mL-1:32), only 2 (5 mL-1:32 and 10 mL-1:32) had replicates that remained positive (Ct < 35) after treatment, but every group had at least 1 suspect (35 ≤ Ct < 40) swab that remained negative on the bioassay. Because PCR tests for PEDV are readily available at the major veterinary diagnostic laboratories, and because bioassays are expensive and difficult to perform, it is tempting to use PCR results as endpoints for disinfection studies. However, there are multiple mechanisms that result in viral inactivation including deterioration of genetic material but also membrane disruption or protein denaturation (16). The results of this study suggest that a positive result for PEDV by RT-PCR on an environmental sample following application of AHP does not necessarily indicate that an infectious dose of live virus remains. This is consistent with results from another study that evaluated a phenol, quaternary ammonium compound, sodium hypochlorite, oxidizing agent, and quaternary ammonium/glutaraldehyde combination, for their ability to inactivate PEDV and reduce the amount of viral RNA detectable by RT-PCR (17).

Rather than perform the experiment on full size livestock trailers or small-scale models of trailers, 15.24 cm × 15.24 cm × 2.54 cm smooth aluminum coupons were used as a model in the present study. The ease with which the coupons can be handled made it possible to contaminate the coupons, perform the treatments, collect the inoculum, and inoculate pigs for the bioassay for all study groups in < 1 d. The model also enabled the investigators to stagger the start time for each treatment group so that the pigs could be inoculated immediately after the inoculum was collected, thereby eliminating the need to attempt to neutralize the AHP disinfectant after the 30-minute contact time. However, livestock trailers used to haul pigs have many different types of surfaces and it is not possible to represent all with a single type of coupon. The sidewalls and gates are generally smooth with varying angles that would be represented by the 90° angles where the sidewall meets the bottom surface of the coupons used in this study. The floor of livestock trailers used to haul swine frequently have a raised diamond plate pattern. There are also typically corners and crevices, and surfaces that are perpendicular to the ground where the AHP foam would be drawn away by gravity more quickly. The inability of the coupons to represent all of the surfaces of livestock trailers used to haul swine is a limitation of this study.

The experimental unit for the bioassay was the individual pig. Elevated tubs with solid dividers completely separating each of the 4 pigs from one another were used to house the pigs. The tubs were designed to prevent any contact with neighboring pigs or with
feces from neighboring pigs. Strict biosecurity procedures were followed, but because all of the pigs in a single treatment group were housed in the same room there was a perceived risk that pigs not infected by PEDV in the inoculum could become infected by lateral transmission from one pig in a room to another by study personnel or aerosol transmission. Recent research demonstrated that aerosol transmission did not occur from pigs experimentally infected with PEDV to sentinel pigs housed in close proximity and in the same air space (18). However, in another study, it was shown that PEDV can become airborne and remain infectious while suspended in air (19). To evaluate this risk, a transmission control group was added to this study. One pig in the tub was inoculated with feces that were positive for PEDV while the other 3 were inoculated with feces that were negative for PEDV. During sampling procedures, animals were handled in alternating orders, so that the negative animals were handled after handling the positive animal within the group. In this way, the biosecurity practices were also tested. The pig inoculated with the positive feces became infected, while the other 3 remained negative to PEDV for the 7-day duration of the study. The results suggest that the housing system and animal handling procedures were effective at preventing lateral transmission between pigs by direct contact, humans, or aerosol. The results help demonstrate the validity of the housing model and associated biosecurity practices for this study and future PEDV studies using a swine bioassay.

The formulation of Accel used in this study was registered for use in the United States, contains 4.25% AHP and was labeled against multiple viruses at dilutions of 1:16 to 1:64 with 5 min of contact time. Evaluation of the efficacy of AHP for inactivating PEDV in the presence of feces was conducted at lower dilution rates of AHP (1:16 and 1:32), at room temperature and with coupons on a flat surface during contamination and treatment to mimic the surfaces of a livestock trailer. Further study of the efficacy of AHP under less favorable conditions, including higher dilution rates, colder temperatures, drier and thicker fecal contamination, and with coupons representative of more challenging surfaces of livestock trailers is merited.

Acknowledgments

Funding for this study was provided by Virox Technologies Inc., Oakville, Ontario and Ogena Solutions, Stoney Creek, Ontario. The authors thank AMVC Management Services for in-kind support and students and staff of the Swine Medicine Education Center at Iowa State University for assistance with live animal work.

References

Concurrent vaccination of boars with type 1 and type 2 porcine reproductive and respiratory syndrome virus (PRRSV) reduces seminal shedding of type 1 and type 2 PRRSV
Jiwoon Jeong, Changhoon Park, Ikjae Kang, Su-Jin Park, Chanhee Chae

Abstract

The objective of the present study was to determine the effect of concurrent vaccination of boars with type 1 and type 2 porcine reproductive and respiratory syndrome virus (PRRSV) on seminal shedding of both genotypes. The boars tolerated well concurrent administration of 2 commercial PRRSV vaccines, and no adverse reactions were observed. No interference in the humoral immune response (measured as the level of anti-PRRSV antibodies) or the cell-mediated immune response (measured as the level of PRRSV-specific interferon-γ-secreting cells) was observed after concurrent administration compared with single administration of the same vaccines. Concurrent vaccination significantly reduced the load of type 1 and type 2 PRRSV in blood and semen after singular (type 1 or type 2) and dual (type 1 and type 2) PRRSV challenge, and it did not significantly affect the efficacy of each vaccine. The results demonstrate that concurrent vaccination of boars with type 1 and type 2 PRRSV reduces shedding of both genotypes in semen.

Introduction

Porcine reproductive and respiratory syndrome virus (PRRSV) is an enveloped, single-stranded, positive-sense RNA virus that belongs to the genus Arterivirus, family Arteriviridae, within the order Nidovirales, which also includes equine arteritis virus, lactate dehydrogenase-elevating virus, and simian hemorrhagic fever virus (1). Two genotypes of PRRSV are prevalent, as shown by genetic analysis: type 1 (European) and type 2 (North American). The 2 genotypes share only 55% to 70% nucleotide identity (2–4). In addition, pathogenic differences between types 1 and 2 PRRSV have been described (5).

This virus has become one of the most important viral pathogens for the global swine industry, resulting in immense economic losses due to reproductive failure in breeding females and respiratory disease in growing pigs (6). It can also infect male reproductive organs (7,8), the manifestations in the boars being loss of libido and alterations in semen quality, including a decrease in sperm motility and an increase in the frequency of morphologic anomalies, including an abnormal acrosome (9). Infected boars have been found to shed PRRSV in semen for as few as 4 d and as many as 92 d after experimental infection (10). In semen the virus is transmissible to sows (11–14). Therefore, freedom of semen from PRRSV is a critical issue for commercial boars because artificial insemination (AI) is widely and routinely used in the global swine industry.

Cross-protection of boars by vaccination against heterogenotypic PRRSV is limited. Vaccination of boars with type 1 PRRSV was unable to reduce seminal shedding of type 2 PRRSV after challenge and vice versa (15,16). Although PRRSV-free semen can really only be guaranteed from a PRRSV-free herd and not from PRRSV-vaccinated ones, concurrent vaccination of boars with type 1 and type 2 PRRSV reduces seminal shedding of both genotypes in semen.
herds, the importance of vaccination of boars against PRRSV is to reduce the amount of seminal shedding of PRRSV because the seminal transmissibility of PRRSV is dependent on the viral load (17). Theoretically, vaccination of boars with both type 1 and type 2 PRRSV may be necessary to reduce the seminal shedding of both genotypes efficiently. Hence, the objective of the present study was to determine the effect of concurrent vaccination of boars with type 1 and type 2 PRRSV on seminal shedding of both genotypes.

Table I. Detection of porcine reproductive and respiratory syndrome virus (PRRSV) by real-time polymerase chain reaction in serum and semen from 5 boars per group

<table>
<thead>
<tr>
<th>Group</th>
<th>Virus genotype</th>
<th>Number of boars with positive results in serum/in semen; number of days after challenge</th>
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<tr>
<td>Vac1-2/Ch1-2</td>
<td>Type 1</td>
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<tr>
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</tr>
<tr>
<td>Vac1/Ch1</td>
<td>Type 2</td>
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</tr>
<tr>
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<td>Type 2</td>
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</tr>
<tr>
<td>UnVac/Ch2</td>
<td>Type 1</td>
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<td>Type 2</td>
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<td>Type 1</td>
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</tr>
<tr>
<td>UnVac/UnCh</td>
<td>Type 2</td>
<td>0/0 0/0 0/0 0/0 0/0 0/0 0/0 0/0 0/0 0/0 0/0 0/0 0/0 0/0</td>
</tr>
</tbody>
</table>

Vac1-2/Ch1-2 — vaccinated with type 1 and type 2 PRRSV and then challenged with type 1 and type 2 PRRSV; Vac1-2/Ch1 — vaccinated with type 1 and type 2 PRRSV and then challenged with type 1 PRRSV; Vac1-2/Ch2 — vaccinated with type 1 and type 2 PRRSV and then challenged with type 2 PRRSV; Vac1/Ch1 — vaccinated with type 1 PRRSV and then challenged with type 1 PRRSV; Vac2/Ch2 — vaccinated with type 2 PRRSV and then challenged with type 2 PRRSV; UnVac/Ch1-2 — challenged with type 1 and type 2 PRRSV without prior vaccination; UnVac/Ch1 — challenged with type 1 PRRSV without prior vaccination; UnVac/Ch2 — challenged with type 2 PRRSV without prior vaccination; and UnVac/UnCh — neither vaccinated nor challenged.

Inocula

Type 1 PRRSV (SNUVR090485; pan-European subtype 1) and type 2 PRRSV (SNUVR090851; lineage 1) were used as inocula. The SNUVR090485 virus [GenBank: National Center for Biotechnology Information, Bethesda, Maryland, USA] no. JN315686] was isolated from lung samples from an aborted fetus and a weaned pig in a 1000-sow herd in southwestern Kyunggi Province (18). The SNUVR090851 virus [GenBank no. JN315685] was isolated from lung samples from different newly weaned pigs and from lymph node samples from an aborted fetus in a 1000-sow herd in Chungcheong Province in 2009 (19).

Experimental design

At 8 mo of age, 45 purebred male Landrace pigs were purchased from a PRRSV-free commercial farm. All boars were negative for PRRSV according to the commercial PRRSV enzyme-linked immunosorbent assay (ELISA) HerdChek PRRS X3 Ab (IDEXX Laboratories, Westbrook, Massachusetts, USA) before delivery and on arrival. All boars were individually housed in separate experimental rooms equipped with air conditioning and high-efficiency particulate air filtration to avoid possible transmission of the pathogen between groups throughout the experiment in the research facility.

Porcilis PRRS (lot D353A07; MSD Animal Health, Summit, New Jersey, USA) was used as the type 1 PRRSV vaccine (Vac1) and Ingelvac PRRS MLV (lot 245-659A; Boehringer Ingelheim Vetmedica, St. Joseph, Missouri, USA) was used as the type 2 PRRSV vaccine (Vac2). Sample size was calculated assuming a 90% power (1 − β = 0.90) of detecting a difference at the 5% level of significance (α = 0.05), which was based on expected results for virus load in semen and serum as determined by real-time polymerase chain reaction (RT-PCR) (20). The boars were divided into 9 groups (5 boars per group) by means of the Excel random number generation function (Microsoft Corporation, Redmond, Washington, USA) (Table I).

The boars in the Vac1-2/Ch1-2, Vac1-2/Ch1, and Vac1-2/Ch2 groups were vaccinated intramuscularly with the 2 vaccines at the same time, the type 1 vaccine in the left side of the neck and the type 2 vaccine in the right side of the neck, each dose being 2.0 mL. The boars in the Vac1/Ch1 group were vaccinated intramuscularly with the type 1 PRRSV vaccine (left side of the neck, 2.0 mL), and the boars in the Vac2/Ch2 group were vaccinated intramuscularly with type 2 PRRSV vaccine (right side of the neck, 2.0 mL).

Five weeks after vaccination (vaccination being at −35 d after challenge), the boars in the Vac1-2/Ch1-2 and UnVac/Ch1-2 groups were inoculated intranasally with 1 mL of tissue culture fluid containing 10^5 50% tissue culture infective doses (TCID_{50})/mL of type 1 PRRSV (SNUVR090485, 2nd passage in alveolar macrophages) and 1 mL of tissue culture fluid containing 10^5 TCID_{50}/mL of type 2 PRRSV (SNUVR090851, 2nd passage in MARC-145 cells). The boars in the Vac1-2/Ch1, Vac1/Ch1, and UnVac/Ch1 groups were inoculated intranasally with 1 mL of tissue culture fluid containing 10^5 TCID_{50}/mL of type 1 PRRSV (SNUVR090485, 2nd passage in alveolar macrophages). The boars in Vac1-2/Ch2, Vac2/Ch2, and
UnVac/Ch2 groups were inoculated intranasally with 1 mL of tissue culture fluid containing 10<sup>6</sup> TCID<sub>50</sub>/mL of type 2 PRRSV (SNUV/9090851, 2nd passage in MARC-145 cells). The boars in the UnVac/UnCh group were used as negative controls and were not exposed to vaccine or virus. After PRRSV inoculation the physical condition of the boars was monitored daily and the rectal temperature taken daily. Blood samples were collected from each pig by jugular venipuncture at −35, −14, 0, 7, 14, 21, 28, 35, 42, 49, and 56 d after challenge and tested with the HerdChek PRRS X3 Ab ELISA. All of the methods had been approved by the Seoul National University Institutional Animal Care and Use and Ethics Committee.

**Enzyme-linked immunospot (ELISPOT) assay**

The numbers of PRRSV-specific interferon-γ-secreting cells (IFN-γ-SCs) were determined in peripheral blood mononuclear cells (PBMCs) as previously described (21,22) with some modifications. Briefly, 100 μL containing 5 × 10<sup>5</sup> PBMCs in RPMI 1640 medium supplemented with 10% fetal bovine serum (HyClone Laboratories, Logan, Utah, USA), nonessential amino acids (1 mM; Invitrogen, Carlsbad, California, USA), sodium pyruvate (1 mM; Invitrogen), 2-mercaptoethanol (5 mM), penicillin (50 000 IU/L), and streptomycin (50 μg/mL) was seeded onto plates that had been coated with IFN-γ monoclonal antibody against porcine antigen (10 μg/mL; MABTECH, Mariemont, Ohio, USA) and incubated overnight at 4°C. The cells were stimulated with challenge type 1 or type 2 PRRSV (live virus) in RPMI 1640 medium for 20 h at 3°C in 5% humidified CO<sub>2</sub>; the linear response was tested at multiplicities of infection (ratios of the number of virions added per cell during infection to the number of cells) between 0.01 and 0.1. Phytohemagglutinin (10 μg/mL; Roche Diagnostics GmbH, Mannheim, Germany) and culture medium were used as positive and negative controls, respectively. The wells were washed 5 times with phosphate-buffered saline (PBS; 0.01M, pH 7.4, 200 μL per well). Thereafter, the procedure was conducted with the commercial ELISPOT Assay Kit (MABTECH) according to the manufacturer’s instructions. The spots on the membranes were read by an automated ELISPOT Reader (AID ELISPOT Reader, AID GmbH, Strassberg, Germany). The results were expressed as the numbers of IFN-γ-SCs per million PBMCs.

**Quantification of PRRSV RNA**

RNA was extracted, as previously described (23), from raw semen and blood collected at −35, −21, −7, 0, 7, 14, 18, 21, 25, 28, 32, 35, 39, 42, 46, 49, and 56 d before challenge from all the boars. Real-time PCR for the vaccine and challenge type 1 and type 2 PRRSV was used to quantify PRRSV genomic cDNA copy numbers with the RNA extracted from semen and serum as previously described (22,23).

**Virus isolation**

With the use of alveolar macrophages for type 1 PRRSV and MARC-145 cells for type 2 PRRSV, PRRSV was isolated, as previously described (24,25), with slight modification, from raw semen collected at −35, −7, 0, 7, 14, 28, 42, and 56 d before challenge. Dilution of semen (26), an extensive washing method (27), and increased blind passage were used to reduce the cytotoxicity of the semen and increase the sensitivity of virus isolation. Briefly, 1 mL of boar semen was centrifuged for 20 min at 625 × g. The cell fraction was resuspended in Eagle’s Minimum Essential Medium (EMEM) containing 5% fetal calf serum (FCS) and 2% antibiotics, then diluted 10 and 100 times in EMEM containing 5% FCS and 2% antibiotics. Next, 10<sup>6</sup> cells in RPMI medium were seeded in microplate wells (6-well cell culture plates) and incubated for 1 h with 480 μL of semen diluted 10 and 100 times. The inoculum was then removed and the microplates were washed 3 times with PBS before fresh medium was added and the incubation continued for 5 d. The microplates were then frozen at −70°C. After thawing, 200 μL of the supernatant was blindly passed to fresh cell monolayers. Incubation and blind passage were done 3 times. At each passage the cells were screened for the presence of PRRSV antigens by immunoperoxidase monolayer assay (IPMA) with SR-30 monoclonal antibodies (Rural Technologies, Brookings, South Dakota, USA) against the nucleocapsid protein of PRRSV. Sequencing was done on the purified reverse transcription-PCR products of amplified open reading frame 5 (28).

**Statistical analysis**

Continuous data (for PRRSV RNA, PRRSV serologic findings, and PRRSV-specific IFN-γ-SCs) were analyzed with 1-way analysis of variance (ANOVA) for each time point separately. If the ANOVA showed a significant effect, Tukey’s multiple-comparison test was done at each time point. Pearson’s correlation coefficient was used to assess the relationship of PRRSV RNA load between blood and semen. A P-value of less than 0.05 was considered significant.

**Results**

The boars in the 3 unvaccinated, challenged groups exhibited a slightly increased rectal temperature (39.5°C to 39.8°C) from 3 to 7 d after challenge. The boars in the 5 vaccinated, challenged groups and in the unvaccinated, unchallenged group were clinically normal in health and rectal temperature (38.2°C to 39.4°C) throughout the experiment (Figure 1).

Anti-PRRSV antibodies were detected 2 wk after vaccination (−21 d after challenge) in the serum samples from the 5 vaccinated, challenged groups. In the unvaccinated, challenged boars no anti-PRRSV antibodies were detected in the serum samples until challenge (0 d after challenge), and thereafter these antibodies were detected. Regardless of the commercial PRRSV vaccines used, the vaccinated, challenged boars exhibited significantly higher (P < 0.05) anti-PRRSV antibody values than the unvaccinated, challenged boars −21 to 35 d after challenge (Figure 2). There were no significant differences in anti-PRRSV antibody values between the single-vaccinated (Vac1/Ch1 and Vac2/Ch2) and dual-vaccinated (Vac1-2/Ch1-2, Vac1-2/Ch1, and Vac1-2/Ch2) boars before or after challenge. As expected, no anti-PRRSV antibodies were detected in the serum of the negative-control (UnVac/UnCh) boars throughout the experiment.

After challenge with type 1 PRRSV the mean frequencies of type 1 PRRSV-specific IFN-γ-SCs in the Vac1-2/Ch1-2, Vac1-2/Ch1, and Vac1/Ch1 groups remained at basal levels (< 20 cells/10<sup>6</sup> PBMCs) until −21 d after challenge, when they reached an average of 47.8 ± 9.15 cells/10<sup>6</sup> PBMCs; the mean frequencies in the same 3 groups then decreased to an average of 38.8 ± 10.7 cells/10<sup>6</sup> PBMCs at 0 d after challenge and were significantly higher (P < 0.05)
The mean frequencies of type 2 PRRSV-specific IFN-γ-SCs in the Vac1-2/Ch1-2, Vac1-2/Ch2, and Vac2/Ch2 groups remained at basal levels until -21 d after challenge, when they reached an average of $49 \pm 12.9$ cells/$10^6$ PBMCs; the mean frequencies in the same 3 groups then decreased to an average of $27.2 \pm 7$ cells/$10^6$ PBMCs at 0 d after challenge and were

than those in the UnVac/Ch1-2 and UnVac/Ch1 groups at 7, 14, and 21 d after challenge (Figure 3A). There were no significant differences in the frequencies between the single-vaccinated (Vac1/Ch1) and dual-vaccinated (Vac1-2/Ch1-2 and Vac1-2/Ch1) boars before or after challenge. The mean frequencies remained at basal levels in the unvaccinated, unchallenged group throughout the experiment.

After challenge with type 2 PRRSV the mean frequencies of type 2 PRRSV-specific IFN-γ-SCs in the Vac1-2/Ch1-2, Vac1-2/Ch2, and Vac2/Ch2 groups remained at basal levels until -21 d after challenge, when they reached an average of $49 \pm 12.9$ cells/$10^6$ PBMCs; the mean frequencies in the same 3 groups then decreased to an average of $27.2 \pm 7$ cells/$10^6$ PBMCs at 0 d after challenge and were
significantly higher \((P < 0.05)\) than those in the UnVac/Ch1-2 and UnVac/Ch2 groups at 7, 14, and 21 d after challenge (Figure 3B). There were no significant differences in the frequencies between the single-vaccinated (Vac2/Ch2) and dual-vaccinated (Vac1-2/Ch1-2 and Vac1-2/Ch2) boars before or after challenge. The mean frequencies remained at basal levels in the unvaccinated, unchallenged group throughout the experiment.

Genomic copies of the vaccine and challenge type 1 and type 2 viruses were not detected in the serum from any boar at −35 d after challenge, but, regardless of the vaccine used, genomic copies of the vaccine strains were detected in the serum in all 5 vaccinated groups at −21 d after challenge (14 d after vaccination). Thereafter, no vaccine virus was detected in the serum from the boars in these groups.

Genomic copies of type 1 PRRSV were detected in the serum of the boars challenged with type 1 PRRSV. The boars in groups Vac1-2/Ch1-2, Vac1-2/Ch1, and Vac1/Ch1 had significantly lower numbers \((P < 0.05)\) of genomic copies of type 1 PRRSV in their serum compared with the boars in groups UnVac/Ch1-2 and UnVac/Ch1 at 7 to 21 d after challenge (Figure 4A). Before as well as after challenge there were no significant differences in numbers of genomic copies

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**Figure 3.** Mean frequencies of type 1 PRRSV (A) — and type 2 PRRSV (B) — specific interferon-\(\gamma\)-secreting cells \((\text{IFN-}\gamma\text{-SCs})\) per \(10^6\) peripheral blood mononuclear cells \((\text{PBMCs})\) from the 8 challenged groups. Variation and asterisks as in Figures 1 and 2.
of type 1 PRRSV between the boars vaccinated with a single strain (Vac1/Ch1) and those vaccinated with the 2 strains (Vac1-2/Ch1-2 and Vac1-2/Ch1).

Genomic copies of type 2 PRRSV were detected in the serum of the boars challenged with type 2 PRRSV. The boars in groups Vac1-2/Ch1-2, Vac1-2/Ch2, and Vac2/Ch2 had significantly lower numbers ($P < 0.05$) of genomic copies of type 2 PRRSV in their serum compared with the boars in groups UnVac/Ch1-2 and UnVac/Ch2 at 7 to 21 d after challenge (Figure 4B). Before as well as after challenge there were no significant differences in numbers of genomic copies of type 2 PRRSV between the boars vaccinated with a single strain (Vac2/Ch2) and those vaccinated with the 2 strains (Vac1-2/Ch1-2 and Vac1-2/Ch2).

The prevalence of viremia in the boars is summarized in Table 1. No type 1 PRRSV was isolated from the serum of any boar challenged with type 2 PRRSV and vice versa. No type 1 or type 2 PRRSV RNA was detected in the blood of the negative-control (UnVac/UnCh) pigs throughout the experiment.

Genomic copies of the vaccine and challenge viruses were not detected in the seminal samples from any boar at −35 d after

Figure 4. Mean numbers of genomic copies of type 1 (A) and type 2 (B) PRRSV RNA in serum from the 8 challenged groups. Variation and asterisks as in Figures 1 and 2.
challenge, but, regardless of the vaccine used, genomic copies of the vaccine strains were detected in the semen in all 5 vaccinated groups at −21 d after challenge (14 d after vaccination). Thereafter, no vaccine virus was detected in the semen from the boars in these groups.

Genomic copies of type 1 PRRSV were detected in the semen of the boars challenged with type 1 PRRSV. The boars in groups Vac1-2/Ch1-2, Vac1-2/Ch1, and Vac1/Ch1 had significantly lower numbers ($P < 0.05$) of genomic copies of type 1 PRRSV in their semen compared with the boars in groups UnVac/Ch1-2 and UnVac/Ch1 at 7 and 10 d after challenge (Figure 5A). Before as well as after challenge there were no significant differences in numbers of genomic copies of type 1 PRRSV between the boars vaccinated with a single strain (Vac1/Ch1) and those vaccinated with the 2 strains (Vac1-2/Ch1-2 and Vac1-2/Ch1).

Genomic copies of type 2 PRRSV were detected in the semen of the boars challenged with type 2 PRRSV. The boars in groups Vac1-2/Ch1-2, Vac1-2/Ch2, and Vac2/Ch2 had significantly lower numbers ($P < 0.05$) of genomic copies of type 2 PRRSV in their semen compared with the boars in groups UnVac/Ch1-2 and UnVac/Ch2 at 7 to 14 d after challenge (Figure 5B). Before as well as after challenge

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**Figure 5.** Mean numbers of genomic copies of type 1 (A) and type 2 (B) PRRSV RNA in semen from the 8 challenged groups. Variation and asterisks as in Figures 1 and 2.
Table II. Isolation of PRRSV from the semen of 5 boars per group

<table>
<thead>
<tr>
<th>Group</th>
<th>Virus genotype</th>
<th>Number of boars with positive results; number of days after challenge</th>
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<tr>
<td>Vac1-2/Ch1-2</td>
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</tr>
<tr>
<td>UnVac/Ch1</td>
<td>Type 1</td>
<td>0 4 4 1 0 0</td>
</tr>
<tr>
<td>UnVac/Ch2</td>
<td>Type 2</td>
<td>0 4 4 0 0 0</td>
</tr>
<tr>
<td>UnVac/UnCh</td>
<td>Type 1</td>
<td>0 0 0 0 0 0</td>
</tr>
<tr>
<td></td>
<td>Type 2</td>
<td>0 0 0 0 0 0</td>
</tr>
</tbody>
</table>

Discussion

The results of this study demonstrate that concurrent vaccination of boars with type 1 and type 2 PRRSV is able to reduce shedding of both genotypes in the semen (28). Vaccinated boars shed the 2 vaccine viruses only during the first 21 d after vaccination. These results are similar to those of previous studies (29,30). Seminal shedding of PRRSV plays a major role in the transmissibility of the virus. The transmission of PRRSV via semen to offspring by AI has been reported (31). The impact of semen that is contaminated with PRRSV can be enormous since AI is widely and routinely used in the swine industry. In Korea more than 90% of sows are bred by AI, and more than 80% of swine producers purchase semen from commercial AI centers (C. Chae. personal communication with Korea Pork Producers Association) (http://www.pigtech.co.kr). There are concerns about whether the efficacy of a particular PRRSV vaccine in boars can be affected by another PRRSV vaccine concurrently administered. No interference in the humoral and cell-mediated responses, as measured by the levels of anti-PRRSV antibodies and PRRSV-specific IFN-γ-SCs, was observed in this study after concurrent administration of 2 PRRSV vaccines when compared with single administration of the same vaccines to boars at 8 mo of age. In contrast, a previous study found interference in the induction of type 2 PRRSV-specific IFN-γ-SCs after concurrent administration of 2 PRRSV vaccines when compared with single administration of the same vaccines to 4-week-old pigs (34). We have no clear explanation for this discrepancy, but it may be due to age-dependent immune responses against PRRSV. Adult boars had higher frequencies of induction of IFN-γ-SCs in our current study than did the 4-week-old pigs in the previous study (34). Age-dependent immune responses were observed in another study: mature sows had more abundant IFN-γ-SCs than did 3-week-old piglets or finisher pigs aged 16 to 20 wk after experimental PRRSV infection (33). These results suggest that boars have a better acquired cellular immune response compared with piglets.

The importance of PRRSV vaccine is in reducing the amount of seminal shedding of PRRSV because the seminal transmissibility of PRRSV is dependent upon the viral load (17). Reduction of viral shedding in semen may be related to the cell-mediated immune responses induced by the vaccine. Despite seemingly contradictory results (34), reduction of seminal virus shedding coincided with the appearance of PRRSV-specific IFN-γ-SCs in the present study. Therefore, we believe that induction of PRRSV-specific IFN-γ-SCs by the PRRSV vaccine is one of the main factors leading to reduction of seminal virus shedding in infected boars.

To our knowledge, this is the first study that has evaluated the concurrent vaccination of boars with both genotypes of PRRSV. No interference in the efficacy of either vaccine due to concurrent administration was observed by immunologic and virologic analyses. The vaccines were administered at separate anatomic sites rather than at a single site to avoid any interference between the 2 vaccine viruses. The boars tolerated the vaccines well, and no adverse reactions were observed with concurrent administration.

Using semen from PRRSV-negative boar studs is the primary step in preventing the introduction of new strains into pig herds. Because PRRSV shedding in semen is intermittent and difficult to detect, semen testing is not totally satisfactory for monitoring. Vaccination of boars with both type 1 and type 2 PRRSV is an alternative method of reducing the shedding of PRRSV in semen when previously negative boars are unexpectedly infected with PRRSV in PRRSV-positive herds. Nevertheless, although boar vaccination is acceptable in positive herds, it is never an option in negative herds, and will not guarantee PRRSV-free semen. Seminal shedding of PRRSV was evaluated in the vaccinated, challenged boars until day 56 in the present study. However, the virus can be detected until day 96 after challenge (10). Therefore, the results of this study cannot be extrapolated to the long-term, and further study is needed to evaluate the seminal shedding of PRRSV in vaccinated, challenged boars beyond day 56.

Acknowledgments

This research was supported by contract research funds of the Research Institute for Veterinary Science, College of Veterinary Medicine, and by the BK 21 Plus Program from College of Veterinary
References


Effect of the probiotic Lactobacillus murinus LbP2 on clinical parameters of dogs with distemper-associated diarrhea

Luis Delucchi, Martín Fraga, Pablo Zunino

Abstract

The objective of this study was to assess the effect of the probiotic Lactobacillus murinus native strain (LbP2) on general clinical parameters of dogs with distemper-associated diarrhea. Two groups of dogs over 60 d of age with distemper and diarrhea were used in the study, which was done at the Animal Hospital of the Veterinary Faculty of the University of Uruguay, Montevideo, Uruguay. The dogs were treated orally each day for 5 d with the probiotic or with a placebo (vehicle without bacteria). Clinical parameters were assessed and scored according to a system specially designed for this study. Blood parameters were also measured. Administration of the probiotic significantly improved the clinical score of the patients, whereas administration of the placebo did not. Stool output, fecal consistency, mental status, and appetite all improved in the probiotic-treated dogs. These results support previous findings of beneficial effects with the probiotic L. murinus LbP2 in dogs. Thus, combined with other therapeutic measures, probiotic treatment appears to be promising for the management of canine distemper-associated diarrhea.

Introduction

Research on the use of probiotics, defined as living microorganisms that when administered in adequate amounts confer a health benefit on the host, may lead to promising results for the control of infectious diseases in animals (1,2). These microorganisms have been successfully used for the treatment of diarrhea associated with different causes, such as antibiotic treatment, viral or bacterial infection, travel (“traveller’s diarrhea”), and chemotherapy (3). Most of these organisms have been obtained from feces or from the gut mucosa of healthy individuals (1). This approach is supported by the results of several studies proposing that native probiotic strains would be well-adapted to the target ecologic niche and could successfully compete in that environment (4). Probiotics can influence the gut environment and the epithelial barriers in different ways since they exert effects on junction stability between epithelial cells, mucus production, mucosal immune response, and even commensal or pathogenic microorganisms (5).

Lactobacillus murinus is one of the dominant lactobacilli in the intestinal microbiota of the domestic dog (Canis lupus familiaris) (6). In the present study an L. murinus strain (LbP2) was used for the treatment of distemper-associated diarrhea in dogs. In previous studies in our laboratory this strain had shown antimicrobial activity against Escherichia coli and Clostridium perfringens (7). In addition, enteric persistence of LbP2 had been observed in an in-vivo trial in dogs in which it was also observed that the composition of the dogs’ intestinal microbiota as well as blood and body parameters were not affected (8). As well, L. murinus LbP2 significantly influenced the immune response: the production of total canine fecal immunoglobulin A (IgA) significantly increased after oral administration (9). This could reflect the ability of the probiotic to modulate the immune response at a mucosal level, a potential key factor in the resolution of diarrhea with various causes (10).

Canine distemper, which is distributed worldwide, is caused by a morbillivirus of the family Paramyxoviridae and was originally described in the 19th century. The host spectrum of this virus...
includes numerous families of the *Carnivora* order, including *Canidae* (dogs, foxes, and dingoes), *Procyonidae* (raccoons), *Mustelidae* (ferrets and mink), and *Felidae* (large felids) (11). At the beginning of the disease, dogs are depressed, anorexic, and febrile. The infection then provokes a variety of clinical problems, affecting the gastrointestinal and respiratory tracts and the skin and causing immunosuppression and demyelinating leukoencephalitis (11), as well as catarrhal enteritis with depletion of the Peyer’s patches (12).

The aim of the present work was to assess the effect of orally administered *L. murinus* LbP2 on clinical parameters of dogs with distemper-associated diarrhea.

### Materials and Methods

#### Animals

Nineteen dogs with distemper treated at the Animal Hospital of the Veterinary Faculty, University of Uruguay, Montevideo, Uruguay, were included in the study. Besides clinical examination, the disease was diagnosed by means of a *Canine distemper virus* (CDV) test kit involving the use of nasal or ocular discharges and a sandwich lateral-flow immunochromatograph (Isu Abxis Company, Seoul, Korea). According to the manufacturer, the sensitivity and the specificity of the test are 100% and 98%, respectively. The samples were taken with a swab and diluted in the buffer provided by the manufacturer. Then 3 drops were placed in the cassette for the diagnostic procedure. All the dogs included in the study showed distemper-positive results according to the test interpretation.

All procedures were approved by the Veterinary Faculty’s Bioethics Committee and carried out under the current national ethical regulations (Law 18.611, approved by the Uruguayan Parliament March 28, 2014). The dogs were more than 60 d old (the oldest was aged 8 y) and had diarrhea and other digestive signs. The exclusion criteria were any of poor clinical status, severe weakness, bad nutritional status, and depressed mental status, as well as treatment with antibiotics, probiotics, steroids, or other drugs in the previous 72 h. The dogs aged 8 y (14).

The study was designed as a randomized controlled trial; 13 dogs were to be treated with *L. murinus* LbP2 and the other 7 dogs with a placebo.

#### Probiotic and placebo preparation and administration

*Lactobacillus murinus* LbP2 was grown on MRS agar from stock cultures that had been stored at −80°C (Becton, Dickinson and Company, Franklin Lakes, New Jersey, USA); some colonies were picked and cultured in MRS broth. Both incubations were done for 24 h under microaerophilic conditions at 37°C. After 3 washes in sterile phosphate-buffered saline, bacteria were inoculated in 10% autoclaved skim milk (Becton, Dickinson and Company) for a final bacterial dose of 5 × 10⁹ colony-forming units and lyophilized with a FreezeZone Stopping Tray Dryer (Labconco, Kansas City, Missouri, USA). The vials were stored at 4°C, and contamination and viability controls were done at weeks 3 and 6. The placebo consisted of 10% autoclaved skim milk only, which was lyophilized and stored under the same conditions as the probiotic. Probiotic and placebo were rehydrated with 5 mL of sterile water and administered orally for 5 d, a single dose per day.

#### Clinical assessment

A clinical score based on 5 items was designed. It included the objective signs of stool output and consistency, along with vomiting, and the subjective signs of appetite and mental status (alert or depressed) (13). The normal value for each item was 1; 0 was assigned when the condition was altered. Normal stool output was considered to be fewer than 3 deposits per day. Stool consistency was scored as 0 (watery) to 5 (solid). These 5 items were chosen because of their frequency among clinical signs at presentation in the digestive form of the disease according to a retrospective study of canine distemper cases at the Veterinary Hospital over 13 y (14).

Blood samples were taken from the cephalic vein on days 0 and 6 and analyzed at the Clinical Laboratory of the Veterinary Faculty for hematocrit, hemoglobin level, total leukocyte count, and concentrations of total plasma proteins and albumin.

#### Data analysis

The Wilcoxon test was used to compare the clinical scores within the 2 groups of dogs, and the Mann–Whitney U-test was used to compare the scores between the 2 groups. Student’s *t*-test was used to compare the hematologic parameters between the 2 groups. Differences were considered significant when *P*-values were < 0.05.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Probiotic (12)</th>
<th>Placebo (7)</th>
<th>P-value for the difference in score between those times</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mental status</td>
<td>Before 3 After 5</td>
<td>Before 1 After 1</td>
<td>0.0253</td>
</tr>
<tr>
<td>Appetite</td>
<td>Before 1 After 5</td>
<td>Before 0 After 1</td>
<td>0.0455</td>
</tr>
<tr>
<td>Stool consistency</td>
<td>Before 1 After 5</td>
<td>Before 0 After 1</td>
<td>0.0455</td>
</tr>
<tr>
<td>Stool output</td>
<td>Before 4 After 7</td>
<td>Before 1 After 2</td>
<td>0.0832</td>
</tr>
<tr>
<td>Vomiting</td>
<td>Before 7 After 8</td>
<td>Before 4 After 2</td>
<td>0.6547</td>
</tr>
<tr>
<td>Total</td>
<td>Before 16 After 30</td>
<td>Before 6 After 7</td>
<td>0.0277</td>
</tr>
</tbody>
</table>

**Table I. Clinical scores for dogs with distemper-associated diarrhea treated with the probiotic *Lactobacillus murinus* LbP2 or with placebo**
**Table II. Hematologic values for the dogs before and after treatment**

<table>
<thead>
<tr>
<th>Variable</th>
<th>Treatment; time of measurement; group mean ± standard deviation*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Probiotic</td>
</tr>
<tr>
<td></td>
<td>Before</td>
</tr>
<tr>
<td></td>
<td>Before</td>
</tr>
<tr>
<td>Hematocrit (%)</td>
<td>37.88 ± 7.65</td>
</tr>
<tr>
<td>Hemoglobin concentration (g/dL)</td>
<td>11.89 ± 2.95</td>
</tr>
<tr>
<td>Total plasma protein concentration (g/dL)</td>
<td>6.76 ± 3.04</td>
</tr>
<tr>
<td>Albumin concentration (g/dL)</td>
<td>4.38 ± 1.86</td>
</tr>
<tr>
<td>Leukocyte count (per mm³)</td>
<td>9505 ± 4313</td>
</tr>
</tbody>
</table>

* For all the variables the difference between the mean values obtained before and after treatment was not significant ($P > 0.05$).

**Results**

In the group to be treated with the probiotic, 1 dog died 72 h before the start of treatment. Of the remaining 12 dogs 7 improved according to the general clinical score and 1 dog worsened; in the other 3 dogs the score did not change. Score variation over time was statistically significant ($P = 0.0277$) (Table I). Of the 7 dogs treated with the placebo, 2 improved slightly according to the clinical score and 2 worsened; in the other 3 dogs the values did not change. No significant differences were observed between the scores before and after treatment in the placebo group ($P = 0.70546$) (Table I).

Significant differences were observed between the clinical scores of the 2 groups ($P = 0.0357$). The score items that influenced the final $P$-value were stool consistency ($P = 0.0455$), mental status ($P = 0.0253$), and appetite ($P = 0.0455$). Although the effect of treatment on stool output was not significant, a clear tendency ($P = 0.0832$) could be seen.

No significant differences in hematologic parameters between initial and final values were observed in either group during the course of treatment (Table II).

**Discussion**

In recent years the use of probiotics has become a promising tool for the treatment and prevention of different diseases, including infections of varied cause, and their use in companion animals has progressively increased (15). Probiotic properties are strain-specific and vary within the same bacterial species (16). Therefore, it is necessary to thoroughly characterize the properties of the different strains that are potentially associated with therapeutic value.

In this study an *L. murinus* strain extensively characterized by our group was used as a therapeutic support for the treatment of dogs with distemper-associated diarrhea. It had already been reported that different *L. murinus* strains showed interesting properties as probiotics with *in vitro* approaches (7,17). Although the use of probiotics has consistently increased in recent years in both humans and animals, there have been few studies evaluating the use of probiotics in cases of canine diarrhea. Herstadet al (18) conducted a controlled clinical trial that included 36 dogs with diarrhea treated with a commercial probiotic based on *Lactobacillus* spp., *Pediococcus* spp., and *Bacillus* spp. of different origins. The authors observed that the time between the start of the treatment and the last abnormal fecal deposit was significantly reduced in the treated group compared with the group that received the placebo.

In our study *L. murinus* LbP2 showed a beneficial effect on the clinical condition of dogs with distemper, probably exerting its effects on the intestinal barrier and immunity. In a previous study we proved that this strain significantly induced the production of enteric IgA (9). This effect shows the ability of the probiotic to modulate the immune response, so it is possible that it also influences enteric inflammation, improving absorption of nutrients and contributing to appetite stimulation and food intake. In this study appetite stimulation was seen in the dogs treated with the probiotic but not in those treated with the placebo.

In a previous work no significant differences were found among the main groups of enteric bacteria of dogs treated with *L. murinus* LbP2 or with placebo (8). Although further studies are required, we could consider that the probiotic effects are not related to an influence of the principal groups in the gut microbiota.

In this study the hematologic parameters of the 2 groups of dogs did not show any significant difference related to treatment. This can be taken as an advantage in the use of *L. murinus* LbP2 since alterations in hematologic parameters, especially the blood leukocyte profile, could be an indicator of risk associated with its use. Clinical use and safety of these live microorganisms in humans and animals are under debate (19). Our results confirm previous findings that *L. murinus* LbP2 did not show any deleterious effects in canines (8).

It is also important to consider that *L. murinus* LbP2 maintained its therapeutic value as a probiotic even after lyophilization since in some cases this preservation procedure can affect a probiotic’s effects (20).

As far as we know, there are no published reports on the use of probiotics for the management of diarrhea associated with canine viruses. However, Benyacoub et al (21) reported that feeding growing dogs a dry food supplemented with a live probiotic enhanced long-term immune functions, including increased induction of specific antibodies by a live-attenuated vaccine against CDV. Studies that included the use of probiotics for the treatment of children with rotavirus-associated diarrhea and acute diarrhea found a reduced number of days of diarrhea with probiotic supplementation (21). Specifically, in children affected by rotavirus, the use of *Saccharomyces boulardii* reduced the time to the resolution of vomiting and diarrhea and the time of hospitalization in comparison with the control patients (22).
In canines, the treatment of secretory diarrhea is a therapeutic challenge because there is no etiologic treatment in most cases, and when there is specific treatment it is lengthy, prolonging the animal’s state of weakness. Viral diseases such as canine distemper occur with these types of signs. Also, as diarrhea is a common sign of many diseases, the indiscriminate use of antibiotics can favor the emergence of resistant microorganisms, aggravating the clinical picture by removing the native microbiota or causing overgrowth of pathogenic bacteria. Considering all these factors and taking into account the results of the present study, the use of probiotics appears to be a promising tool for the management of diarrhea, particularly diarrhea associated with canine distemper.

References

**Lactobacillus casei regulates differentiation of Th17/Treg cells to reduce intestinal inflammation in mice**

Kai Wang, Hao Dong, Yu Qi, Zhihua Pei, Shushuai Yi, Xiaojie Yang, Yanli Zhao, Fanxing Meng, Shouping Yu, Tiezhong Zhou, Guixue Hu

**Abstract**

In order to study the ability of *Lactobacillus casei* to ameliorate murine enteritis, 18 mice were randomly divided into 3 groups: the enteritis group, intervention group, and control group. The interleukin (IL)-6 and transforming growth factor-β (TGF)-β content in mouse peripheral blood and duodenum was detected using an enzyme-linked immunosorbent assay (ELISA). The number of CD4+CD25+Foxp3+ T-regulatory cells (Tregs) and CD4+IL-17A+ Th17 cells in the mesenteric lymph nodes (MLN) and spleen were detected using flow cytometry, and quantitative reverse transcription polymerase chain reaction (PCR) and western blot analysis were used to measure Foxp3 and retinoid-related orphan receptor-γ (RORyt) mRNA and protein expression in the MLN. Histological changes in the duodenum were observed. Results indicate that in the intervention group, IL-6 content in mouse peripheral blood and duodenum was significantly lower than in the enteritis group (P < 0.05), while TGF-β content was significantly increased compared to the enteritis group (P < 0.05). For the intervention group, the percentages of CD4+CD25+Foxp3+ Tregs in spleen and MLN were increased (P < 0.05), while the percentages of CD4+IL-17A+ Th17 cells were decreased compared to the enteritis group (P < 0.05). The expression of Foxp3 mRNA and protein in the intervention group was higher than in the enteritis group, while RORyt mRNA and protein were significantly lower (P < 0.05). After mice in the enteritis group were treated with *L. casei*, duodenal inflammation was relieved. This study demonstrated that *L. casei* could have possible implications for the enterotoxigenic *Escherichia coli* (ETEC) induced intestinal inflammation by regulating the ratio imbalance of Th17/Treg cells.

**Résumé**

Afin d'étudier la capacité de *Lactobacillus casei* à soulager l'entérite murine, 18 souris ont été réparties de manière aléatoire en trois groupes : le groupe entérite, le groupe intervention, et le groupe témoin. Les quantités d'interleukine (IL)-6 et de facteur de croissance transformant β (FCT-β) dans le sang périphérique et le duodénum de souris furent détectées à l'aide d'une épreuve immunoenzymatique (ELISA). Les cellules T régulatrices (Tregs) CD4+CD25+Foxp3+ et les cellules CD4+IL-17A+ Th17 dans les nœuds lymphatiques mésoentériques (NLM) et la rate ont été détectées et dénombrées par cytométrie en flux, et réaction d'amplification en chaîne quantitative avec la transcriptase réverse, et l'analyse par immunobuvardage fut utilisée afin de mesurer l'expression de l'ARNm et de la protéine Foxp3 et du récepteur orphelin γ apparenté au rétinoïde (RORyt) dans les NLM. Les changements histologiques dans le duodénum ont été observés. Les résultats indiquent que dans le groupe intervention, le contenu en IL-6 dans le sang périphérique et le duodénum était significativement moindre que dans le groupe entérite (P < 0.05), alors que le contenu en FCT-β était augmenté de manière significative comparativement au groupe entérite (P < 0.05). Pour le groupe intervention, les pourcentages de Tregs CD4+CD25+Foxp3+ dans la rate et le NLM étaient augmentés (P < 0.05), alors que les pourcentages de cellules CD4+IL-17A+ Th17 étaient diminués comparativement au groupe entérite (P < 0.05). L'expression de l'ARNm et de la protéine Foxp3 dans le groupe intervention était plus élevée que dans le groupe entérite, alors que l'expression de l'ARNm et de la protéine RORyt était significativement moindre (P < 0.05). Suite au traitement des souris du groupe entérite avec *L. casei*, l'inflammation duodénale était résorbée. La présente étude a démontré que *L. casei* pourrait avoir des implications possibles dans l'inflammation intestinale induite par *Escherichia coli* entérotoxinogène en régulant le débalancement du ratio de cellules Th17/Treg.

(Traduit par Docteur Serge Messier)
Enterotoxigenic *Escherichia coli* (ETEC) strains are a major cause of diarrheal disease in humans and animals (1). Enterotoxigenic *E. coli* can produce heat-labile (LT) and heat-stable (ST) toxins, which stimulate fluid and electrolyte secretion from intestinal cells leading to diarrhea (2). Antibiotics have been widely applied in diarrhea prevention to improve animal health and benefit the animal industry economy. Due to the extensive veterinary use and potential to cause selective proliferation of antibiotic resistant bacteria, antibiotic use has recently raised increased concerns (3). As an alternative to antibiotics, probiotics have captured the public’s attention and considerable research is being conducted on these microorganisms. One such probiotic, *Lactobacillus casei*, can tolerate enzyme activity in the oral cavity, gastric acid, and intestinal bile acids and can thus survive in the intestinal tract, maintain intestinal flora balance, enhance animal immune capacity, and promote animal growth and development (4).

Th17 cells are proinflammatory cells that secrete inflammatory factors such as interleukin (IL)-17A. Interleukin-17A is involved in various inflammatory diseases, autoimmune diseases, and acute transplant rejection reactions (5). Retinoid-related orphan receptor-γ (RORγt) is a specific transcriptional regulator required for Th17 cell differentiation (6). One of the important functions of regulatory T-cells (Tregs) is the immunosuppressive regulation of auto-reactive T-cells (7), and they play a critical role in immune self-tolerance. Forkhead transcription factor (Foxp3) is predominantly expressed in CD4+CD25+ Treg cells and is a master regulator for the development and function of Treg cells (8). Regulatory T-cells are important in the development and outcome of various diseases, including cancer, infectious diseases, and transplant immunity (9,10). Regulatory T-cells can release anti-inflammatory cytokines IL-10 and transforming growth factor-β (TGF-β) to depress chronic inflammation. Th17 and Treg cells can differentiate in the thymus at the stage of CD4 single-positive thymocytes (11), and IL-6 and TGF-β are the key cytokines that determine their respective cell fates (12). Interleukin-6 promotes Th17 cells differentiation by inducing STAT3-dependent activation of RORγt (13), while TGF-β signaling is required for the induction of Foxp3 expression (14). Their dynamic equilibrium can maintain the body’s immune response at appropriate levels. Therefore, setting the boundaries of mediation and regulation of inflammatory reactions, Treg/Th17 balance, distinct from the number and function of each subset alone, is an imperative checkpoint in immune homeostasis. In this study, we used *L. casei* to interfere with the ETEC-mediated mouse duodenal inflammation model that had previously been developed (15), the aim was to study the mechanism by which *L. casei* interferes with enteritis by analyzing the Treg/Th17 balance.

## Materials and methods

### Bacterial strain and animals

*Lactobacillus casei* ATCC393 and ETEC K88 C83912 were provided by the Institute of Microbiology, College of Animal Science and Technology, Jilin Agricultural University, China. Male specific pathogen free (SPF) BALB/c mice, 6 to 8 wk of age, were purchased (Beijing HFK Bioscience Company, Beijing, China). The mice were housed in polystyrene cages with stainless steel wire lids and given water and food *ad libitum*. The housing was maintained at a constant temperature (21°C to 22°C) with a 12-hour light-dark cycle. The protocol was approved by the Committee on the Ethics of Animal Experiments of the Jilin Agricultural University, China.

### Experimental design

Eighteen mice were randomly divided into 3 groups: the enteritis group, intervention group, and control group. Six mice were assigned to each group, the experiments were repeated 3 times. Firstly, the intervention group was administered 0.2 mL of 2.0 × 10^8 colony-forming units (cfu)/mL *L. casei* for 7 d. Second, the mice in all 3 groups were pre-treated with 0.2 mL of 1% NaHCO_3_. After 30 min, the intervention and enteritis groups were administered 0.2 mL of 2.0 × 10^8 cfu/mL ETEC K88 daily for 3 d. The control group was administered the same dose of normal saline. Mice were euthanized on the 15th day.

### Enzyme-linked immunosorbent assay (ELISA) analysis of IL-6 and TGF-β

On the 15th day, the mice were euthanized and peripheral blood, duodenum, and orbital venous blood samples from each mouse from each group were collected and serum was isolated. The duodenum was weighed and homogenized with cold saline and centrifuged at 7378 × g at 4°C for 10 min to get the supernatant. The levels of IL-6 and TGF-β in the serum and duodenum of each mouse were determined using an ELISA kit (Lengton Bioscience Company, Shanghai, China), according to the manufacturer’s instructions.

### Flow cytometric analysis of CD4+CD25+Foxp3+ Tregs and CD4+IL-17A+ Th17 cell

The spleen and mesenteric lymph nodes (MLN) were aseptically isolated from each mouse, homogenized in 1 mL dulbecco’s modified eagle media (DMEM), and centrifuged 5 min (4°C, 295 × g). Cells in the spleen were split using red blood cell lysis buffer (Becton, Dickinson and Company, New Jersey, America). Cells were then washed twice and suspended in flow cytometry staining buffer. Viable cells were enumerated using a hemocytometer with trypan blue exclusion. Briefly, to measure the percentage of Tregs, prepared cells (1 × 10⁶) were washed by centrifugation in flow cytometry staining buffer. Then, cells were immersed in the buffer for 30 min at 4°C. Next, cells were fixed and permeabilized in a fixation/permeabilization solution for 30 min and subsequently stained using 0.5 μg of phycoerythrin (PE) rat anti-mouse Foxp3. For analysis of Th17 cells, the cell suspension was stained using phorbol myristate acetate (PMA) 50 μL (25 ng/mL), ionomycin 20 μL (1 μg/mL), and brefeldin A 0.7 μL (Sigma-Aldrich, St. Louis, Missouri, USA) in 24-well plates. After 4 h of culturing (37°C, 5% CO₂), the cells were transferred to tubes and washed twice in phosphate-buffered saline (PBS). Then the cells were re-suspended with 1 × permeability buffer and incubated for 40 min at 4°C in the dark, and washed twice in wash buffer. Cells were stained using 10 μL of PE rat anti-mouse IL-17A at 4°C for 1 h. Finally, the cells were washed using 1 × wash buffer and analyzed by flow cytometry. At least 10 000 cells per sample were assayed using a flow cytometry apparatus (FACS Calibur flow...
cytometry apparatus; Becton Dickinson, California, USA) and analyzed using computer software (FlowJo7.6; Becton, Dickinson and Company, New Jersey, USA).

**Quantitative real-time reverse transcription polymerase chain reaction (RT-PCR) analysis of Foxp3 and RORγt**

Total RNA was isolated from mesenteric lymph nodes samples of 5 mice from each group with an RNA extraction kit (Bio-flux RNA Extraction Kit; BioFlux Company, Tokyo, Japan) and was subsequently reverse transcribed (PrimeScript 1st Strand cDNA Synthesis Kit; BioFlux Company). The expression levels of FoxP3, RORγt, and GAPDH were determined using Synergy Brands Premix Ex Taq™ II (SYBR® Premix Ex Taq™ II; TaKaRa, Dalian, China) based on the manufacturer’s instructions. The primer sequences are shown in Table I. Briefly, 2.0 μL of the cDNA template was added to a total volume of 25.0 μL containing 12.5 μL of SYBR Premix Ex Taq II; TaKaRa, Dalian, China), 8.5 μL of double distilled water (ddH2O) and 0.5 μL of the forward and reverse primers. The thermal cycling conditions were as follows: i) pre-denaturation (30 s at 95°C), and ii) amplification and quantification (40 cycles of 5 s at 95°C, 30 s at 60°C). The relative expression was defined as the ratio of the target gene to the housekeeping gene using the formula 2^(-ΔΔCt).

Relative expression was normalized and expressed as a ratio to the expression in the control group.

**Western blot analysis of Foxp3 and RORγt**

Mesenteric lymph nodes were carefully isolated and crushed. Total protein was extracted using bicinchoninic acid (BCA) protein quantification kit (Keygen Biotech Company, Nanjing, China) according to the manufacturer’s instructions. Protein concentration was detected using the BCA protein detection kit (Biouniquer Technology Company, Nanjing, China), and 50 μg of protein lysate from each sample was used for further analyses. After sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), the protein was electro-transferred to a polyvinylidene fluoride (PVDF) membrane. Next, 5% skim milk powder was used to block non-specific antibody binding to the membrane for 1 h. Foxp3, RORγt, and lamin B primary antibodies (eBioscience, California, USA) were added, and samples were incubated overnight at 4°C. Samples were washed 3 times using tris-buffered saline and tween 20 (TBST) and horseradish peroxidase (HRP)-conjugated secondary antibodies were added. Samples were then incubated at room temperature for 1 h and then washed by TBST 3 times. Finally, the blots were visualized using enhanced chemiluminescent (ECL) kits (Amersham, Buckinghamshire, UK).

### Table I. Quantitative reverse transcription polymerase chain reaction (qRT-PCR) primer sequences

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Forward primer (5’-3’)</th>
<th>Reverse primer (5’-3’)</th>
<th>Fragment length (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Foxp3</td>
<td>5’-TGCCTCAAGAGACTTGGA-3’</td>
<td>5’-GGCATTTGTTCTGAGAG-3’</td>
<td>86</td>
</tr>
<tr>
<td>RORγt</td>
<td>5’-CAGCAGTGAATGGGCTACTCCT-3’</td>
<td>5’-TGTTGCACTTTGACTCTC-3’</td>
<td>19</td>
</tr>
<tr>
<td>GAPDH</td>
<td>5’-CACTCACGCACAATTCAAAGGCAC-3’</td>
<td>5’-GACTCCACGACATACGAC-3’</td>
<td>14</td>
</tr>
</tbody>
</table>

bp — base pairs

**Figure 1.** The levels of interleukin (IL)-6 and transforming growth factor-β (TGF-β) in different groups of mice. A — TGF-β content in mouse peripheral blood and duodenum was determined by ELISA from the different groups; B — IL-6 content in mouse peripheral blood and duodenum was determined by ELISA from the different groups. Control: control group, the mice were administered normal saline. Group A: enteritis group, the mice were administered 0.2 mL 2.0 × 10^8 cfu/mL ETEC K88 daily for 3 d. Group B: intervention group, the mice were administered 2.0 × 10^8 cfu/mL *L. casei* for 7 d, then 2.0 × 10^8 cfu/mL ETEC K88 for 3 d. Results are expressed as means ± SD of the 6 mice in each group.
**Histological analysis of the duodenum**

For the histological examination, the entire duodenum was excised and cleaned using normal saline, one component (1 to 2 cm) was fixed in 10% neutral buffered formalin and embedded in paraffin. Four micrometer thick longitudinal sections were cut and stained with hematoxylin and eosin (H&E).

**Statistical analyses**

The results are presented as the mean ± SD. Statistical analysis was done using computer software (SPSS 18.0; International Business Machines Corporation, New York, America). Data were analyzed using 1-way analysis of variance (ANOVA) and Tukey’s multiple comparison test was used to determine significance levels between groups. The results were considered significant at a 95% confidence level ($P < 0.05$).

**Results**

**Interleukin-6 and TGF-β content**

All T-cells depend on cytokines for their growth and survival. Interleukin-6 and TGF-β are the key cytokines required to induce...
Th17 and Tregs phenotypes, respectively. In order to study the differentiation of the native T-cells, the levels of IL-6 and TGF-β in mouse peripheral blood and duodenum from different groups were detected, the results showed that IL-6 content in mouse peripheral blood and duodenum for the *L. casei* intervention group was significantly lower than in the enteritis group \((P < 0.05)\), while TGF-β content was significantly increased \(P < 0.05\) (Figure 1).

**Changes in CD4⁺CD25⁺Foxp3⁺ Tregs and CD4⁺IL-17A⁺ Th17 cell percentages after *L. casei* intervention**

The Tregs and Th17 cells have opposite effects on autoreactivity and inflammation. The percent difference between Tregs and Th17 cells in the spleen and MLN from different groups was studied. The results showed that the percentages of CD4⁺CD25⁺Foxp3⁺ Tregs and CD4⁺IL-17A⁺ Th17 cells in the spleen lymphocytic population for the control group were 0.696 ± 0.071% and 1.243 ± 0.104%, respectively, and 0.644 ± 0.0747% and 1.738 ± 0.149% in MLN. For the enteritis group, the percentages of CD4⁺CD25⁺Foxp3⁺ Tregs and CD4⁺IL-17A⁺ Th17 cells were 0.667 ± 0.0861% and 2.170 ± 0.319%, respectively, in spleen lymphocytic population, and 0.473 ± 0.0436% and 2.496 ± 0.213% in MLN. For the intervention group, the percentages of CD4⁺CD25⁺Foxp3⁺ Tregs and CD4⁺IL-17A⁺ Th17 cells were 1.0767 ± 0.1831% and 1.461 ± 0.168%, respectively, in spleen lymphocytes, and 1.507 ± 0.203% and 1.123 ± 0.224%, respectively, in MLN (Figures 2A, 2C). There were significant differences in the percentages of CD4⁺CD25⁺Foxp3⁺ Tregs and CD4⁺IL-17A⁺ Th17 cells when comparing the intervention and enteritis groups \((P < 0.05)\) (Figures 2B, 2D).

**Quantitative PCR and western blot analysis of Foxp3 and RORγt**

The RORγt is an important transcription factor for the differentiation of Th17 cells, whereas Foxp3 is the master transcription factor for Tregs. The relative mRNA and protein expression of Foxp3 and RORγt in the MLN was analyzed. The results showed that the expression of Foxp3 mRNA and protein for the intervention group was significantly higher than in the enteritis group \((P < 0.05)\), while the expression of RORγt mRNA and protein was lower than the enteritis group \((P < 0.05)\) (Figure 3).

**Histological analysis**

Compared to the control group (Figure 4A), intestinal villi disintegrated and fractured in the enteritis group. There were increased numbers of goblet cells in the mucosal layer, the intestinal lumen was filled with inflammatory secretions, and the gut gland gap was widened. Fluid had leaked from the gap and resulted in edema (Figure 4B). For the intervention group, inflammation was relieved and the numbers of goblet and inflammation cells decreased (Figure 4C).

**Discussion**

The Th17 cells promote inflammatory reactions by secreting IL-17. The Treg cells are an important component of immune regulation and immunosuppression, and CD4⁺CD25⁺ Tregs are the Treg population that have been most widely studied. These cells play an important role in inflammatory diseases, allergies, and induction of transplantation tolerance (16). "Probiotics" is the general term for live microorganisms that are beneficial to host organisms and can improve resistance to and prevent disease. At present, it has been shown that probiotics can be used to treat antibiotics induced diarrhea, inflammatory bowel disease (IBD), irritable bowel syndrome, and other bowel diseases (17). Probiotics can prevent toxins from entering the circulation, suppress translocation of pathogenic bacteria to the intestinal mucosa, and prevent inflammation (18,19). Probiotics are increasingly widely applied in the management of enteritis, but its mechanism of action is not clear to date. Thus, this experiment aimed to study effects of *L. casei* on Th17 and Treg cells.
The Th cell precursors differentiate into naive T-cells under antigenic stimulation, and naive T-cells can differentiate into various auxiliary T-cell subsets under the influence of various cytokines, antigens, and antigen-presenting cells. Naive T-cells will preferentially express the transcription factor, RORγt, under the influence of IL-6 and TGF-β to become Th17 cells (20), or will preferentially express the transcription factor FoxP3 under the influence of IL-10 and TGF-β to become Tregs (12). Through in vivo experiments, this study showed that IL-6 expression in the intervention group was lower than in the enteritis group, indicating that L. casei induced naive T-cells to differentiate toward Treg cells.

The Th17 and Treg cells are closely related in both differentiation and function. Under physiological conditions, inflammatory responses promoted by Th17 cells are in equilibrium with those suppressed by Tregs, while their imbalance is associated with infectious diseases (21,22), autoimmune diseases (23,24), and cancer (25,26). Pertinent studies have shown that RORγt/Foxp3 are key regulatory factors in the development and maturation of Th17 and Treg cells (27–30), and play an important role in maintaining their functions. Further, IL-17A/IL-10 are the main effector molecules of Th17/Treg cells (31–33). This study found that after mice with enteritis were administered L. casei, the number of CD4+CD25+Foxp3+ Tregs and expression of Foxp3 mRNA and protein were all significantly increased. Naive T-cells were induced to differentiate toward Treg cells. Through detection of morphological changes in intestinal histology, it was determined that intestinal inflammation was relieved. This study suggests that the dynamic expression of Th17 and Treg cells was closed associated with occurrence and outcome of intestinal inflammation.

The balance of Th17/Treg cells can regulate immune responses to both self and foreign antigens and can underlie the pathogenesis and pathophysiological changes of certain inflammatory and autoimmune diseases. This study showed that by correcting the ratio imbalance of Th17/Treg cells, L. casei could have possible implications for the ETEC induced intestinal inflammation. This study adds interesting information about the growing field of probiotics and commensal microbiota.

**Acknowledgment**

This work was supported by the China Natural Science Foundation (No. 31372413).

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Inflammatory bowel disease affects density of nitrergic nerve fibers in the mucosal layer of the canine gastrointestinal tract

Andrzej Rychlik, Sławomir Gonkowski, Marcin Nowicki, Jarosław Calka

Abstract

The objective of this study was to determine the effect of inflammatory bowel disease (IBD) on the density of nitrergic nerve fibers in the mucosal layer of different sections of the gastrointestinal tract of dogs. Twenty-eight German shepherd hybrid dogs of both sexes, weighing from 15 to 25 kg and aged 6 to 10 y, were studied. The dogs were divided into 4 groups with 7 animals in each group: healthy animals, as well as dogs suffering from mild, moderate, and severe IBD. Immunoreactivity to neuronal isoform of nitric oxide synthase, which is a marker of nitrergic neurons, in samples of the mucosal layer in the duodenum, jejunum, and descending colon was studied using the single immunofluorescence method and the number of nerve fibers was evaluated in each observation field. The obtained results showed that IBD causes an increase in the number of nitrergic nerve fibers in all intestinal segments studied and these changes are directly proportional to the intensity of the disease process. These observations may be useful in diagnostic evaluation of the stage of canine inflammatory bowel disease in veterinary practice. The pathological mechanisms of these observed changes and the specific reasons for them are still not completely explained, however, and further study is required.

Résumé

L’objectif de la présente étude était de déterminer l’effet de la maladie inflammatoire de l’intestin (MII) sur la densité des fibres nerveuses nitrergiques dans la couche muqueuse de différentes sections du tractus gastro-intestinal de chiens. Vingt-huit chiens de race Berger Allemand croisée des deux sexes, pesant entre 15 et 25 kg et âgés de 6 à 10 ans, ont fait partie de l’étude. Les chiens ont été répartis en quatre groupes de 7 chiens dans chaque groupe : des animaux en santé, aussi bien que des chiens souffrant de MII légère, modérée et sèvere. L’immunoréactivité à un isoforme neuronal de l’oxyde nitrique synthase, qui est un marqueur des neurones nitrergiques, dans des échantillons de la couche muqueuse du duodénum, jéjunum, et côlon descendant a été étudiée en utilisant la méthode d’immunofluorescence unique et le nombre de fibres nerveuses a été évalué dans chaque champs observé. Les résultats obtenus montrent que la MII cause une augmentation des fibres nerveuses nitrergiques dans tous les segments intestinaux étudiés et que ces changements sont directement proportionnels à l’intensité du processus pathologique. Ces observations pourraient être utiles pour l’évaluation diagnostique des stades de la MII en pratique vétérinaire. Par contre, les mécanismes pathologiques des changements observés et leurs raisons spécifiques ne sont pas encore complètement éclaircis et des études supplémentaires sont requises.

Introduction

The gastrointestinal (GI) tract is innervated by the enteric nervous system (ENS) located in the wall of the esophagus, stomach, and intestine (1,2). There is also extrinsic innervation, in which bodies of neuronal cells supplying the GI tract are situated in parasympathetic, sympathetic, and sensory ganglia (3,4). The ENS and extrinsic innervation system regulate all functions of the GI tract, such as intestinal motility, blood flow, and excretive activity (2). It is well-known that neurons within the ENS and extrinsic neuronal cells supplying the gut may undergo the process of neuronal plasticity, which is reflected by changes within the neurons under various stimuli. These stimuli can be grouped into 2 categories: physiological conditions, such as ontogenesis of the GI tract or diet (5,6), and pathological factors, such as intestinal and extra-intestinal diseases, mycotoxin poisoning, and nerve injury (7,8).

It should be noted that neurons innervating the GI tract show the presence of a wide range of active substances, which may act as neuromediators and/or neuromodulators (2). Changes in the expression of these substances are the main symptom of neuronal plasticity (8). Nitric oxide (NO) is one of many active substances that occur in the intestinal neuronal structures (9). It is a gaseous neurotransmitter, which is synthesized from the guanidine group of L-arginine during reaction, catalyzed by a family of enzymes called nitric oxide synthases (NOSs) (10). Three isoenzymes are recognized here: neuronal (nNOS or NOS-1); cytokine-inducible (iNOS or NOS-2);...
and endothelial (eNOS or NOS-3) (10), among which nNOS is the marker of nitrergic neurons.

Until now, nitric oxide has been described in the intestinal neuronal structures of various parts of the GI tract in a wide range of mammal species including humans (11–15). Most nitrergic neurons have been noted within the myenteric plexus, which is part of the ENS located between the longitudinal and circular muscle layers of the GI tract (12). Most nitrergic nerve fibers have been observed within the muscular layers of the intestine, although mucosal nerves that are immunoreactive to nNOS have also been described (13,14).

Nitric oxide in the GI tract has an inhibitory function, namely it suppresses intestinal motility, both by directly influencing muscular cells and by inhibiting the release of stimulating neuromediators (16). Nitric oxide (NO) takes part in the reduction of excretive action in the GI tract, regulation of blood flow in the intestinal wall (by dilation of blood vessels), as well as neuroprotective and/or adaptive processes in the enteric nervous system (11,17,18). Finally, NO is probably involved in changes of neuronal nitric oxide synthase-like immunoreactivity (nNOS-LI) in intestinal nervous structures, which have been noted in the presence of various pathological factors, such as Crohn’s disease, inflammatory processes, or mycotoxin poisoning (19,20).

Idiopathic inflammatory bowel disease (IBD) in dogs, however, is a group of chronic and recurrent pathological states within the GI tract, which is an important problem in recent veterinary practice. Both the diagnosis of IBD, which consists of ruling out other reasons for intestinal symptoms characteristic of this disease, such as loss of appetite, vomiting, and diarrhea (21–23), and its treatment are difficult. Many pathological mechanisms accompanying IBD, such as the influence of IBD on intestinal nervous structures and changes in expression of neurotransmitters and/or neuromodulators, are not fully explained and knowledge about them is limited (24–26).

The present study reports for the first time on how IBD affects nitrergic nervous structures within the mucosal layer of the canine

| Table I. Clinical CIBDAI scores for groups as well as individual dogs |
|------------------------|--------|------------------------|--------|--------|
| Group                  | CIBDAI score | Patient number | Individual | CIBDAI score | X | SD |
| Control group          | 0 to 3    | 1              | 0          | 0               | 0 |
| Group I — mild IBD     | 4 to 5    | 1              | 4          | 4.43            | 0.57 |
| Group II — moderate IBD| 6 to 8    | 1              | 7          | 7.29            | 0.71 |
| Group III — severe IBD | < 9       | 1              | 9          | 9.57            | 1.43 |

CIBDAI — Clinical Inflammatory Bowel Disease Activity Index.
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digestive tract. Results obtained may not only add to knowledge about the pathological mechanisms of IBD, but could also contribute to improving diagnostic methods for this disease.

**Materials and methods**

Twenty-eight German shepherd hybrid dogs of both sexes, weighing from 15 to 25 kg and aged 6 to 10 y, participated in this study. The control group consisted of 7 healthy dogs approved for the experiment during screening tests for IBD conducted at a dog shelter in Olsztyn, Poland. The experimental groups were selected from patients of the Veterinary Clinic at the University of Warmia and Mazury in Olsztyn, Poland. All experimental animals were approved for the experiment based on results of clinical, laboratory, and endoscopic examinations, as well as histopathology of duodenal, jejunal, and colonic mucosa.

Dogs with IBD were subjected to biochemical, radiological, parasitological, bacteriological, and mycological stool tests and provocation trials in order to exclude other diseases associated with chronic diarrhea. Based on their Clinical Inflammatory Bowel Disease Activity Index (CIBDAI) scores (22), animals suffering from IBD were divided into the following groups: Group I — mild IBD, CIBDAI score — 4 to 5 points, histopathological score “+”; Group II — moderate IBD, CIBDAI score — 6 to 8 points, histopathological score “++”; and Group III — severe IBD, CIBDAI score — 9 to 16 points, histopathological score “+++”. Each group consisted of 7 dogs. The clinical CIBDAI scores for all groups as well as for individual dogs are listed in Table I. All procedures were carried out according to the instructions of the Local Ethical Committee in Olsztyn, Poland (decision number 47/2009/DTN).

Tissue samples were obtained from all dogs, both experimental and control, during gastroscopic or colonoscopic examinations with the use of FB-24U-1 biopsy forceps with a diameter of 2.5 mm and FB-50U-1 biopsy forceps with a diameter of 3.7 mm (Olympus Medical System Corp., Tokyo, Japan). Three biopsy specimens from every section of the gastrointestinal tract from each dog were fixed by immersion in 4% buffered paraformaldehyde solution for 15 min and rinsed in phosphate buffer (pH 7.4) for 3 d. Tissue samples were then transferred to 18% saccharose solution in phosphate buffer and stored at 4°C for at least a week and then frozen (at -22°C), sectioned into slices of 10 μm thickness in the Microm Cryostat HM 525 (Thermo Scientific, Walldorf, Germany), and placed on gelatin-coated slides.

Slices were subjected to routine single immunofluorescence method as described in a previous study (24). In brief, the sections were dried for 45 min at room temperature, rinsed in buffered PBS solution (PBS, 0.1 mol, pH 7.4, 3 × 15 min), and incubated for 1 h with blocking solution containing 10% goat serum, 0.1% bovine serum albumin (BSA), 0.01% sodium azide (NaN₃), Triton X-100, and thimerosal in PBS. After rinsing in NaCl solution, tissue samples were incubated overnight in a humidity chamber at room temperature with primary antibodies directed against the neuronal form of NOS (Rabbit; Cappel, Aurora, Ohio, USA), at a working dilution of 1:4000. The next day, slices were again rinsed in PBS (3 × 15 min) and incubated with specific secondary antibody conjugated to Alexa Fluor 594 at a working dilution of 1:1000 (Donkey; Invitrogen,
Carlsbad, California, USA) for 1 h at room temperature. After repeated rinsing in NaCl solution, the specimens were mounted on slides with glycerol solution and PBS (1:2; pH 7.4) and covered with coverslips.

Tissue samples were observed under the Olympus BX51 fluorescence microscope equipped with appropriate filters and the density of nNOS-like immunoreactive (nNOS-LI) mucosal nerve fibers was defined by a semi-quantitative evaluation based on counting nNOS-positive nerves in the field of view (0.1 mm²). Such fibers were counted in 4 fields of view in 3 sections of every biopsy specimen from the duodenum, jejunum, and colon. A total of 36 fields of view in every intestinal part of each dog was evaluated. Observed fields of view were located at least 100 µm apart, which prevented the repeated counting of the same nerves. Obtained data were pooled and presented as a mean ± standard deviation (SD).

Standard controls of specificity of the method were carried out. Antibody against nNOS was tested by pre-absorption of antiserum with appropriate antigen and omission and replacement of primary antiserum by non-immune serum. The pre-absorption test was done as described in a previous study (13) and included incubation of the intestinal slices with “working” dilutions of antibody directed towards nNOS, which had already been pre-absorbed for 18 h at 37°C with 1.0 mM solution of nitric oxide synthase (Sigma Aldrich, St. Louis, Missouri, USA). These controls completely eliminated specific stainings.

The specificity of the primary antibody was verified by Western blot analysis on canine ileum, which was obtained from another dog euthanized due to cancer at the Veterinary Clinic of the University of Warmia and Mazury in Olsztyn, Poland. Frozen tissue (200 mg) was homogenized in 1800 µL of sodium dodecyl sulfate (SDS) sample buffer (27) using an Ultra-Turrax homogenizer (Janke & Kunkel, IKA Works GmbH & Co. KG, Staufen, Germany). Samples were heated to 95°C for 4 min, cooled on ice, and centrifuged for 5 min at 2000 × g. Supernatant (30 µL) was loaded to 10% SDS-polyacrylamide gel electrophoresis (PAGE) gel (27), together with 5 µL of molecular weight standard (PageRuler Broad Range Protein Ladder, ThermoFisher, USA). Electrophoresis was done for 90 min at 100 V in a Mini-Protein 3 Cell Electrophoresis System (BioRad, Richmond, California, USA). After electrophoresis, the proteins were blotted to polyvinylidene difluoride (PVDF) membrane (BioRad) in Mini Trans-Blot Electrophoretic Transfer Cell (BioRad) using transfer buffer (25 mM TRIS, 192 mM glycine, pH 8.3) at 100 V for 80 min. After transfer, proteins in the blot were stained with 0.05% Amido Black in 10% acetic acid, following destaining in 10% acetic acid-20% methanol. Positions of lanes and molecular weight marker bands were marked on the membrane with a soft pencil and the membrane was rinsed in PBS. The membrane was then blocked with a blocking solution (3% BSA in TBS-0.05% Tween 20) for 1 h at 25°C. The blocking solution was replaced with a primary anti-nNOS antibody, working dilution 1:4000, in a blocking solution (Rabbit; Cappel) and incubated overnight at 25°C.

After incubation, the membrane was washed 3 × 10 min with PBS and then incubated for 90 min in secondary biotinylated antibody solution (VectaStain; Vector Laboratories, Burlingame, California, USA) in the blocking solution. After incubation, the membrane was washed 3 × 10 min in TBS-0.05% Tween and incubated for 40 min with a streptavidin-conjugated alkaline phosphatase (R&D Systems, Minneapolis, Minnesota, USA), diluted 1:500 in TBS-0.05%...
Tween. The membrane was then washed 3 × 10 min in TBS-0.05% Tween and incubated for 5 min with alkaline phosphatase-activation buffer [100 mM NaCl, 5 mM magnesium chloride (MgCl₂), 0.1% Tween 20, 100 mM TRIS, pH 9.5]. The color reaction was developed in the alkaline phosphatase-activation buffer containing 6 μL of BCIP [5-Bromo-4-chloro-3-indolyl phosphate, 20 mg/mL, (Sigma Aldrich)] and 8 μL of Nitroblue tetrazolium (NBT) (50 mg/mL, Sigma Aldrich) per 10 mL. The color reaction was stopped by rinsing in tap water, followed by ultrapure water. The membrane was dried and scanned with a desktop scanner. The image was digitally processed and the locations of selected molecular weight standard bands were placed in the image (Figure 1). The “<” mark in the figure shows the location of a 150 kDa band.

The significance of differences between groups was determined by the Kruskal-Wallis test at \( P \leq 0.05 \) (significant) and \( P \leq 0.01 \) (highly significant). The results were processed using Statistica 9.1 Software (StatSoft, Tulsa, Oklahoma, USA).

### Results

During the present study, nitricergic nerve fibers were noted in all intestinal segments studied, both in control animals and in dogs suffering from IBD, but the population of such fibers was low (Figures 2, 3). In healthy dogs, the average number of nerve processes immunoreactive to nNOS in all studied regions of the intestine fluctuated at around 0.40 per observation field and was even in all intestinal fragments studied (Table II). In individual observation fields, usually 1 or none of the nitricergic nerve was noted (Figures 2A, 3AI, 3AII). Observation fields with 2 such nerve processes were only observed occasionally.

Obtained results show that the density of the mucosal nerve fibers increased in proportion to the intensity of the IBD process (Figures 2, 3, 4). In the duodenum (Figure 2), a gradual increase in the number of nitricergic nerves proportional to the intensity of the disease process was noticed. The average number of nerve processes immunoreactive to nNOS per observation field amounted to 0.48 ± 0.14 in control animals and 0.55 ± 0.21, 0.77 ± 0.22, and 1.26 ± 0.24 in groups I, II, and III, respectively (Table II, Figure 4). In the duodenum of dogs with severe IBD (group III), the population of nitricergic nerves was the most numerous among all intestinal fragments studied. Some observation fields with 3 nNOS-positive nerves were noted only in this group (Figure 2D).

In the jejunum of healthy dogs, the number of nNOS-like immunoreactive nerves was slightly lower than within the duodenum and came to 0.42 ± 0.18 (Table II, Figure 3AI). Mild and moderate IBD (groups I and II) caused a slight increase of such nerves (0.56 ± 0.23 and 0.54 ± 0.07, respectively), whereas in dogs suffering from severe IBD (group III), the increase in the number of described nerve processes was greater and amounted to 0.84 ± 0.15 per observation field (Table II, Figure 4).

Changes observed in the mucosal layer of the descending colon (Figure 3B) were similar to those observed in the duodenum and jejunum. Namely, in control animals, the average number of nNOS-positive nerves was slightly lower than within the duodenum and came to 0.42 ± 0.18 (Table II, Figure 3AI). Mild and moderate IBD (groups I and II) caused a slight increase of such nerves (0.56 ± 0.23 and 0.54 ± 0.07, respectively), whereas in dogs suffering from severe IBD (group III), the increase in the number of described nerve processes was greater and amounted to 0.84 ± 0.15 per observation field (Table II, Figure 4).

Changes observed in the mucosal layer of the descending colon (Figure 3B) were similar to those observed in the duodenum and jejunum. Namely, in control animals, the average number of nNOS-positive nerves was slightly lower than within the duodenum and came to 0.42 ± 0.18 (Table II, Figure 3AI). Mild and moderate IBD (groups I and II) caused a slight increase of such nerves (0.56 ± 0.23 and 0.54 ± 0.07, respectively), whereas in dogs suffering from severe IBD (group III), the increase in the number of described nerve processes was greater and amounted to 0.84 ± 0.15 per observation field (Table II, Figure 4).

Statistical analysis showed essential differences, especially between the control group and group I and dogs with severe IBD.

Figure 3. Distribution pattern of neuronal isoform of nitric oxide synthase-like immunoreactive nerve fibers (arrows) in the mucosal layer of the canine jejunum (I) and descending colon (II). A — physiological conditions; B — moderate inflammatory bowel disease (IBD); C — severe IBD.
Highly statistically significant differences were observed in the duodenum and descending colon of healthy dogs (group C) and dogs with mild IBD (group I) compared to those in dogs with severe IBD (group III). In the case of the jejunum, highly statistically significant differences were observed only between control dogs and those with severe IBD.

In regard to the localization of nerves studied, most nNOS-positive fibers, both in physiological conditions as well as during IBD, were observed in the superficial layer of the mucosa and probably supplied the enterocytes and especially enteroendocrine cells. Considerably fewer nNOS-LI nerves were noted in the deep layer of mucosa, not far from blood vessels.

### Discussion

In spite of the numerous important functions of nitric oxide (NO) in the gastrointestinal tract, the number of nitrergic mucosal nerve fibers observed during the present study seems to be very slight. Values noted herein are lower than those observed in the gastrointestinal tract of other species of mammals (11), which may be a result of interspecies differences. The present study, however, seems to be partially compatible with previous studies, where most nitrergic neuronal structures have been noted first within the intestinal muscular layer and the myenteric plexus of the ENS (11,13,14).

The present results show that canine IBD may change the number of nNOS-positive mucosal nerve fibers in various segments of the digestive tract. Moreover, a directly proportional relationship was noted between the number of these nerves and the intensity of pathological processes connected with inflammatory infiltration of the lamina propria of the mucosal layer.

It should be pointed out that knowledge about IBD-induced changes in the expression of active substances within nerve structures in the canine digestive tract is limited (24,26,28). Both the pathological mechanisms of these observed changes and the specific

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**Table II. The number of NOS-immunoreactive nerve fibers per observation field in the duodenum, jejunum, and descending colon in control dogs (Group C) and in dogs suffering from mild (Group I), moderate (Group II), and severe (Group III) IBD**

<table>
<thead>
<tr>
<th></th>
<th>Group C</th>
<th>Group I</th>
<th>Group II</th>
<th>Group III</th>
</tr>
</thead>
<tbody>
<tr>
<td>Duodenum</td>
<td>0.48 ± 0.14c</td>
<td>0.55 ± 0.21c</td>
<td>0.77 ± 0.22</td>
<td>1.26 ± 0.24a,b</td>
</tr>
<tr>
<td>Jejunum</td>
<td>0.42 ± 0.18c</td>
<td>0.56 ± 0.23</td>
<td>0.54 ± 0.07</td>
<td>0.84 ± 0.15a</td>
</tr>
<tr>
<td>Colon</td>
<td>0.48 ± 0.28c</td>
<td>0.54 ± 0.30c</td>
<td>0.61 ± 0.06</td>
<td>0.97 ± 0.15a,b</td>
</tr>
</tbody>
</table>

*a* Significantly different from control group.

*b* Significantly different from group I.

*c* Significantly different from group III.

Kruskal-Wallis test — $P < 0.05$ — lowercase letters; $P < 0.01$ — uppercase letters.
reasons for them remain unknown. The increase in the number of nNOS-like immunoreactive mucosal nervous structures observed during the present study could be due to factors responsible for triggering canine IBD, which include bacterial, allergic, and environmental agents (21–23). These noted changes could also be due to secondary processes, such as pain or digestive dysfunction, that occur during IBD. The increase in the density of fibers immunoreactive to nNOS may reflect changes in synthase of this enzyme at the transcriptional, translational, or metabolic level, as well as modifications in transport of its molecules from cell bodies to nervous processes.

Observations during the present study may also confirm the findings of previous studies, which suggest that nitric oxide is a neuroprotective neuronal factor that can function in adaptive processes of the nervous system in response to various pathological agents (25,29). This function of NO within the digestive tract is arguable. It is commonly accepted that expression of neuronal neuroprotective factors increases in the presence of other pathological factors. Such changes have been observed both in the present study and in previous investigations of Crohn’s disease or mycotoxin poisoning (19,20). Other pathological processes, such as diabetes mellitus (30), however, caused a decrease in the number of nNOS-immunoreactive intestinal nervous structures. These discrepancies strongly suggest that nitric oxide plays different roles in various pathological processes.

Another reason for changes observed during the present study may be related to inflammatory processes. Until now, it has been well-established that nitric oxide (NO) can have both pro-inflammatory properties, e.g., during arthritis, and anti-inflammatory properties, e.g., during lung inflammatory disorders, depending on the type of inflamed tissues (10). Within the gastrointestinal tract, NO is primarily a pro-inflammatory mediator. It is produced in mucosal neutrophils during acute inflammatory processes, as well as in monocytes and lymphocytes during chronic disease (31). Nitric oxide (NO) also causes an increase in TNF-α concentration, which contributes to infiltration of the mucosal layer by activated neutrophils (32).

Previously mentioned functions of nitric oxide are connected with activity of one of the cytokine-inducible isoenzyme of NOS (iNOS) (10), which among other things, is activated during intestinal inflammatory processes and contributes to damage of the mucosal macrophages (33). This type of NOS, induced by tumor necrosis factor, starts tumor protein p53-dependent processes that result in apoptosis of intestinal epithelium during chronic colitis ulcerosa (34). It is known that inhibitors of iNOS activity significantly reduce the intensity of inflammatory processes and also reduce damage to the mucosal layer of the inflamed intestine (35).

Knowledge about the participation of neuronal isoenzymes of NOS (nNOS) is limited. Previous studies (36) and relatively well-known interactions between the enteric nervous system and immunological structures within the gastrointestinal tract, especially during intestinal diseases (19), suggest that results obtained in the present study may be due to the previously described roles of NO in inflammatory processes. Observed changes, however, may be due to the disturbances of intestinal motility that are often observed during IBD. It is possible that the increase in density of nitrigenic nerves, which play a role in muscular relaxation (11,16), could be the enteric nervous system’s adaptive response to intestinal irritation and diarrhea.

In conclusion, results obtained in the present study show that IBD causes an increase in the number of mucosal nitrigeric nerve fibers in the canine digestive tract. These changes are directly proportional to the intensity of the disease process, which despite the difficulties connected with the small number of mucosal nNOS-LI nerves and relatively high costs and labor intensity of the immunofluorescence technique, could be useful in diagnostic evaluation of the stage of canine inflammatory bowel disease in veterinary practice. The mechanisms of these observed changes and the specific reasons for them are still not completely explained, however, and further study is required.

Acknowledgments

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References


Assessment of regional left ventricular systolic function by strain imaging echocardiography in phenotypically normal and abnormal Maine coon cats tested for the A31P mutation in the MYBPC3 gene

Arine Pellegrino, Alexandre G.T. Daniel, Guilherme G. Pereira, Paula H. Itikawa, Maria Helena M.A. Larsson

Abstract

Myocardial dysfunction occurs in cats with hypertrophic cardiomyopathy (HCM), but little is known about the early stages of the disease. Strain imaging echocardiography is a method that enables the quantitative assessment of myocardial function and deformity, allowing the characterization of systolic dysfunction. The objective of this study was to assess systolic function using strain imaging echocardiography in Maine coon cats genetically tested for the A31P mutation in the MYBPC3 gene, with and without ventricular hypertrophy. For this purpose, 57 Maine coon cats of both genders, with an unknown status regarding the mutation at inclusion, were included prospectively and evaluated by conventional and strain imaging echocardiography. Comparisons were made among cats without hypertrophy (n = 45), suspect cats (n = 7), and cats with hypertrophic cardiomyopathy (n = 5), and also between the heterozygous for the mutation group (n = 26) and the negative for the mutation group (n = 28). Finally, in the group of phenotypically normal cats, heterozygous cats carrying the mutation were compared to cats without the mutation. Strain values were compared among the groups (blinded prospective study). While echocardiography demonstrated normal contractility, strain values (middle of the septum) were lower in HCM cats. Strain values (base of anterior wall of the left ventricle) were lower in heterozygous than in negative cats, even before hypertrophy. Negative correlation was observed between some values of myocardial strain and thickness. While strain imaging echocardiography was able to detect systolic abnormalities, despite apparent normality on conventional echocardiography, it was not able to identify cats that carry the A31P mutation in the MYBPC3 gene. Strain imaging echocardiography could be a useful tool, however, for detecting systolic alterations in HCM cats with an apparently normal systolic function or for detecting alterations in normal carriers of the MYBPC3 gene mutation.

Résumé

Le dysfonctionnement myocardique se produit chez des chats avec une cardiomyopathie hypertrophique (CMH), mais peu de choses sont connues sur les stades initiaux de la maladie. L'échocardiographie par imagerie de déformation (strain imaging) est une méthode qui permet l'évaluation quantitative de la fonction myocardique et de la déformation, permettant ainsi la caractérisation de la dysfonction systolique. L'objectif de la présente étude était d'évaluer la fonction systolique en utilisant l'échographie par imagerie de déformation chez des chats de race Maine Coon testés génétiquement pour la mutation A31P dans le gène MYBPC3, avec et sans hypertrophie ventriculaire. À cette fin, 57 chats Maine Coon des deux sexes, avec un statut inconnu en regard de la mutation au moment de l'inclusion dans l'étude, ont été inclus de manière prospective et évalués par échographie conventionnelle et par imagerie de déformation. Des comparaisons ont été faites parmi les chats sans hypertrophie (n = 45), les chats suspects (n = 7), et les chats avec cardiomyopathie hypertrophique (n = 5), et également entre les hétérozygotes pour le groupe avec mutation (n = 26) et les négatifs pour le groupe avec mutation (n = 28). Finalement, dans le groupe de chats phénotypiquement normaux, les chats hétérozygotes porteurs de la mutation ont été comparés aux chats sans la mutation. Les valeurs de déformation ont été comparées parmi les groupes (étude prospective à l’aveugle). Alors que l’échographie a montré une contractilité normale, les valeurs de déformation (au milieu du septum) étaient plus faibles chez les chats avec CMH. Les valeurs de déformation (à la base de la paroi antérieure du ventricule gauche) étaient plus faibles chez les chats hétérozygotes que chez les chats négatifs, et ce même avant l’hypertrophie. Une corrélation négative fut observée entre quelques valeurs de déformation myocardique et d’épaisseur. Bien que l’échographie par imagerie de déformation était en mesure de détecter des anomalies systoliques, malgré une apparence de normalité lors de l’échographie conventionnelle, elle n’était pas en mesure d’identifier les chats porteurs de la mutation A31P dans le gène MYBPS3. L’échographie par imagerie de déformation pourrait toutefois être un outil utile pour détecter des altérations systoliques chez des chats CMH avec une fonction systolique apparemment normale ou pour détecter des altérations chez des porteurs normaux de la mutation dans le gène MYBPC3.

(Traduit par Docteur Serge Messier)
Introduction

Hypertrophic cardiomyopathy (HCM) is characterized by left ventricular concentric hypertrophy with no evidence of pressure overload or hormonal stimulation as a cause (1–3). It is the main type of cardiomyopathy found in cats and is associated with the development of heart failure, systemic thromboembolism, and sudden death (4). Hypertrophic cardiomyopathy (HCM) is a genetically and phenotypically heterogeneous disease (5,6) and one cause of it in Maine coon cats is the A31P mutation in the MYBPC3 gene (4).

Myocardial thickening and myocardial collagen deposition (interstitial and replacement fibrosis) increase ventricular stiffness (7). The increase in left ventricular stiffness results in an increase in diastolic pressure for any given diastolic volume (8–10). The increased filling pressure results in increased atrial, pulmonary venous, and pulmonary capillary pressures (11–13).

Conventional echocardiography is the most commonly used test for evaluating cardiac anatomy and function (14) and is the best noninvasive diagnostic method for differentiating HCM from other cardiomyopathies (2,9,15,16). Two-dimensional, color flow Doppler, and tissue Doppler imaging are used to identify the 2 basic components of HCM (hypertrophy and diastolic dysfunction) and the common sequelae to this disease, e.g., mitral regurgitation and outflow obstruction due to systolic anterior motion (SAM) of the mitral valve and left atrial enlargement (16).

Tissue Doppler imaging (TDI) is a noninvasive diagnostic tool that is capable of evaluating the heart wall motion and permits the quantification of regional and global myocardial function (3,9,17). It is sensitive and specific for evaluating myocardial dysfunction, although as with other methods, it can be influenced by preload and afterload (3,17). It can also assist in detecting abnormalities in cats that are genetically affected by HCM before the development of myocardial hypertrophy (3,4,9). Strain is the amount of deformity of a tissue when a certain amount of force is applied to it (18,19). Measuring strain by tissue Doppler echocardiography enables a quantitative evaluation of regional myocardial function, is sensitive to the changes identified in various diseases in cats and humans (20,21), and aids in the characterization of myocardial heterogeneity in cardiomyopathies in humans (18,22). Strain eliminates the influence of the movement of the whole heart (translational movement) (20,23). Strain has been used in humans to help differentiate the causes of hypertrophy by assessing systolic contraction and lusitropy (24). Human patients with HCM have decreased strain values compared with normal individuals and a negative correlation between strain and the degree of hypertrophy has been noted (19).

While strain echocardiography is new and has rarely been used in cats, it is reliable, provides good repeatability, and enables early detection of systolic dysfunction in certain stages of feline HCM (20). A study conducted in cats in different stages of HCM showed that, although conventional echocardiography demonstrated normal contractility, strain values were lower in animals with hypertrophy, demonstrating that systolic dysfunction is already present in the early stages of the disease (20).

A study comparing strain echocardiography in humans tested for a mutation that causes HCM showed reduced strain values in patients with hypertrophy (18). No differences were observed between carriers of the mutation without hypertrophy and normal patients, which indicates that systolic function remains preserved at this stage, although diastolic dysfunction is already present.

The objective of the present study was to assess systolic function using strain echocardiography in Maine coon cats genetically tested for the A31P mutation in the MYBPC3 gene, with and without ventricular hypertrophy. Before the development of detectable changes on conventional echocardiography, strain alterations in young cats may assist in early diagnosis and increase knowledge of the pathophysiology of the disease.

Materials and methods

Animals

Fifty-seven Maine coon cats of both genders were included in this blinded, prospective study, which was approved by the Bioethics Committee of the University of São Paulo. All animals were subjected to anamnesis, physical examination, and blood work [complete blood (cell) count and serum urea, creatinine, liver enzyme, total protein, albumin, sodium, potassium, total calcium, phosphorus, and total T4 thyroid hormone concentrations]. Electrocardiography, thoracic radiography, and mensuration of arterial blood pressure (by Doppler vascular method) were conducted according to previously established methodologies (25–27).

Animals presenting with systemic arterial hypertension, nephropathy, and/or hyperthyroidism were excluded from the study, as well as cats that presented any other illness that could interfere with the cardiovascular system.

Whole blood samples were sent to the Veterinary Cardiac Genetics Laboratory at Washington State University for identification of the A31P mutation in the MYBPC3 gene (3). Based on the results, the animals were classified as negative, heterozygous, or homozygous (3,28). The authors did not receive the genetic results for the cats until the study had ended in order to reduce subjective interference in interpreting the examinations (blinded study).

Conventional echocardiography

Echocardiographic examination was carried out using a Vivid 7 Expert echocardiograph (General Electric-Standpromaden, Horten, Norway), equipped with software for tissue Doppler echocardiography, as recommended by the Echocardiography Committee of the Specialty of Cardiology, American College of Veterinary Internal Medicine (14) and the American Society of Echocardiography (29). Simultaneous electrocardiographic monitoring was carried out during the echocardiogram, without the use of sedation and/or tranquilization, with cats positioned in left lateral decubitus.

All variables were measured 3 times on 3 different non-consecutive cycles and an average of these 3 measurements was calculated for each variable. The images for the measurement of the LV were acquired in the right parasternal window, transverse section, at the level of insertion of the chordae tendineae on the papillary muscles (M mode). The presence of myocardial hypertrophy was determined when the diastolic thickness of the interventricular septum (IVSd) and/or the free wall of the left ventricle (LVWd) were equal to or greater than 0.6 cm (4,30). Animals with a diastolic
thickness less than 0.5 cm were considered normal and cats with values between 0.5 and 0.6 cm were considered suspect for HCM (4,30,31). Concentric hypertrophy was considered symmetric when the ratio IVSd/LVWd was between 0.7 and 1.3 (4). When asymmetric hypertrophy was observed, segmental hypertrophy was measured using the 2-dimensional mode. The diameters of the aortic root (Ao) and the left atrium (LA) were measured using the 2-dimensional mode (31), right parasternal window, and transverse section at the region of the base of the heart. The left atrium (LA) was considered enlarged when the ratio LA/Ao was greater than 1.5 (20).

The parameters evaluated by pulsed Doppler echocardiography were maximum velocity of aortic flow and pulmonary artery flow, maximum velocity of the transmitral flow waves E and A, E/A ratio, E-wave deceleration time, and isovolumetric relaxation time (IVRT).

After the echocardiographic examination, the animals were classified according to HCM, as normal group, suspect group, or HCM group. Moreover, to assess the animals before the development of ventricular hypertrophy, normal cats were classified according to their genotypes as normal and negative, normal and heterozygous, or normal and homozygous.

**Strain echocardiography**

Strain imaging echocardiography was carried out according to a recent study with cats (20), using the tissue Doppler imaging (TDI) methodology. In the strain mode, longitudinal myocardial function was evaluated using the apical 4-chamber view and the apical 2-chamber view and the radial function was evaluated using a transverse section at the level of the papillary muscles (Figures 1 and 2). Complete digital data from 3 heart cycles were stored in cine loop format for subsequent myocardial strain analysis. The evaluated parameters included: systolic movement (SIS) of the LV at the base and middle region of the IVS and LVW at the apical 4-chamber view (longitudinal); and the base and middle region of the anterior left ventricular wall (ALVW) and posterior left ventricular wall (PLVW) at the apical 2-chamber view (longitudinal) and at the region of the endocardium and epicardium at the level of the papillary muscles on the lateral wall (transverse). Simultaneous electrocardiographic monitoring was carried out during the evaluation, in which the “event marker” tool was used to identify the opening and closing of the aortic valve and instantaneous heart rate was measured to observe the influence of the heart rate on each evaluated measure (correlation between heart rate and value of strain). The repeatability was evaluated for all parameters in the apical 4-chamber view, in the apical 2-chamber view, and in the transversal view by the same echocardiographer (within day variability).

**Statistical analysis**

The Shapiro-Wilk test was used to assess data normality. Mean, standard deviation, minimum value, maximum value, and median were calculated for the variables that presented a normal distribution. The P-value for Student’s t-test and/or for Mann-Whitney test (2 groups) was used to compare the averages of the continuous variables, as well as the P-value for analysis of variance (ANOVA) and/or Kruskal-Wallis test (3 or more groups). Due to the small number of homozygotes, these animals were not considered for statistical analysis. When the normality tests were rejected in at least 1 group or when little data were present, Mann-Whitney and Kruskal-Wallis tests were used instead of the respective t-test and ANOVA. Frequency tables and Pearson’s Chi-square and/or Fisher’s exact test were used for the categorical variables. In order to assess intraobserver variability (repeatability), a paired t-test was used for differences equal to zero, obtaining a coefficient of variation for all views evaluated. The level of significance was fixed at 5%, always considering a 2-tailed alternative hypothesis.

**Results**

**Epidemiological characteristics**

Among the 57 cats, 23 were male (40.4%) and 34 were female (59.6%). Body weight varied from 3.1 to 7.9 kg (5.11 ± 1.26 kg), with an average of 6.20 ± 0.81 kg (4.9 to 7.9 kg) for male cats and
Hypertrophic cardiomyopathy was considered mild in 3 heterozygotes, moderate in 1 homozygote, and severe in 1 heterozygote (Table II).

Regarding genotypic classification, values for the maximum velocity (and pressure gradient) of the aortic flow were greater in heterozygotes than in negative cats ($P < 0.05$). No significant changes were observed for the remaining echocardiographic parameters or for the heart rate (Table II).

As to the phenotypic classification, the diameter of the LA was greater in the HCM group than in normal cats ($P < 0.05$). Statistical differences were noted in the IVRT, which was greater for the HCM group than for the suspect ($P < 0.05$) and normal cats ($P < 0.001$), and in the velocity of aortic flow, which was also greater for the HCM group than for the suspect group ($P < 0.001$) and normal cats ($P < 0.001$) (Table II). Three of the 5 cats with HCM had systolic anterior motion (SAM) of the mitral valve. No significant changes were observed for the remaining parameters.

When evaluating the group of phenotypically normal cats, no statistically significant differences were observed for the different parameters of conventional echocardiography between cats with and without mutation (Table II).

**Strain imaging results**

Strain values in relation to genotype and phenotype are described in Tables III and IV, respectively. On genotypic evaluation, a statistical difference was observed between the strain values obtained on the 2-chamber longitudinal view at the base of the ALVW, with smaller values obtained for heterozygotes compared to those animals that were negative for the mutation ($P = 0.001$). As for phenotypic evaluation, a significant difference was noted for strain values obtained from the apical 4-chamber view, at the middle region of the IVS, with smaller values obtained for the HCM group than for normal animals ($P = 0.01$).

When evaluating only the group of cats without HCM, a statistically significant difference was noted between negative and heterozygous individuals for strain values obtained at the base of the ALVW. Strain values were lower for heterozygous animals than for negative animals ($P = 0.019$) (Table V).

When evaluating all groups, a statistically significant, negative correlation was found between ventricular thickness and strain values ($P < 0.05$; Pearson’s correlation $= -0.27$) at the middle region of the ALVW. There were no correlations between heart rate and strain values.

Regarding intraobserver repeatability (in all evaluated views: 4-chamber, 2-chamber, and transversal), the second measurement was, on average, 0.66 points greater than the first and the standard deviation was greater than 2.

**Discussion**

The evaluated population consisted of Maine coon cats genetically tested for the A31P mutation of the MYBPC3 gene. Among the 57 cats, 51% were positive for the mutation (46% heterozygous and 5% homozygous). According to previous studies (4,20,32,33), this gene mutation is present in approximately 34% of Maine coon cats worldwide, although few studies have correlated the genotype with the occurrence of HCM.

Among the evaluated animals, 8.77% already had HCM and were all positives for the MYBPC3 gene mutation (4 heterozygotes and

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**Table I. Distribution of Maine coon cats according to genotype and phenotype**

<table>
<thead>
<tr>
<th>Classification</th>
<th>No mutation ($n = 28$)</th>
<th>Heterozygous for mutation ($n = 26$)</th>
<th>Homozygous for mutation ($n = 3$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal ($n = 45$)</td>
<td>n = 25</td>
<td>n = 19</td>
<td>n = 1</td>
</tr>
<tr>
<td>Suspects ($n = 7$)</td>
<td>n = 3</td>
<td>n = 3</td>
<td>n = 1</td>
</tr>
<tr>
<td>HCM ($n = 5$)</td>
<td>n = 0</td>
<td>n = 4</td>
<td>n = 1</td>
</tr>
<tr>
<td>Total ($n = 57$)</td>
<td>n = 28</td>
<td>n = 26</td>
<td>n = 3</td>
</tr>
</tbody>
</table>

HCM — hypertrophic cardiomyopathy.

4.37 ± 0.93 kg (3.1 to 6.7 kg) for female cats. The average weight of male cats was significantly higher than that of the female cats ($P < 0.0001$). No significant differences in weight could be noted between intact and spayed/neutered animals. The cats varied in age from 8 to 89 mo (33.72 ± 17.22 mo) and no significant difference was noted between the average ages of the male and female cats. All cats were asymptomatic for cardiovascular disorders by the time of the present study.

**Genotypic and phenotypic classification**

Regarding genotype classification, 28 cats were negative for the mutation (49%; mean age $= 2.61 ± 1.31$ y), 26 were heterozygous (46%; mean age $= 3.00 ± 1.55$ y), and 3 were homozygous (5%; mean age $= 2.98 ± 1.66$ y). Homozygous cats were excluded from the statistical analysis because of the small number. No significant differences were noted in the weight, gender, or age range of the animals.

Regarding phenotypic classification, 45 cats were considered normal (79%; mean age $= 2.82 ± 1.49$ y), 7 suspect (12%; mean age $= 3.10 ± 1.45$ y), and 5 affected by HCM (9%; mean age $= 2.26 ± 0.77$ y). No differences were observed in the gender or age range of the animals, although normal cats had lower body weights than the suspect ($P < 0.001$) and HCM group ($P < 0.05$). The distribution of the cats according to genotype and phenotype is shown in Table I.

With the purpose of investigating early changes (before ventricular hypertrophy), a comparison was made within the group of normal cats. Normal cats were classified according to their genotypes as: normal and negative ($n = 25$; mean age $= 2.54 ± 1.27$ y); normal and heterozygous ($n = 19$; mean age $= 3.27 ± 1.68$ y); and normal and homozygous ($n = 1$; mean age $= 1.33 ± 0.00$ y). Statistical analysis was done between normal negative and normal heterozygous animals. There were no differences in weight, age range, or sex distribution in these groups.

**Conventional echocardiography results**

When evaluating transmitral flow, a fusion of the E and A waves occurred, which prevented the evaluation of 26 animals (45.6%). A positive correlation was observed between the occurrence of wave fusion and heart rate ($P < 0.008$).

The 5 cats in the HCM group presented a symmetric form of the disease, with an IVSd/LVWd ratio ranging from 0.8 to 1.3. Hypertrophic cardiomyopathy was considered mild in 3 heterozygotes, moderate in 1 homozygote, and severe in 1 heterozygote (Table II).

---
### Table II. Mean and standard deviation of conventional echocardiography values in Maine coon cats

<table>
<thead>
<tr>
<th>Parameters</th>
<th>All ( (n = 57) )</th>
<th>Negatives ( (n = 28) )</th>
<th>Heterozygous ( (n = 26) )</th>
<th>Homozygous ( (n = 03) )</th>
<th>Normal ( (n = 45) )</th>
<th>Suspects ( (n = 07) )</th>
<th>HCM ( (n = 05) )</th>
<th>Normal negatives ( (n = 25) )</th>
<th>Normal heterozygous ( (n = 19) )</th>
</tr>
</thead>
<tbody>
<tr>
<td>HR (bpm)</td>
<td>197.8 ± 31.5</td>
<td>193.86 ± 32.89</td>
<td>202.5 ± 30.3</td>
<td>194.4 ± 35.5</td>
<td>201.8 ± 32.5</td>
<td>186.2 ± 25.1</td>
<td>178.4 ± 21.7</td>
<td>196.0 ± 32.9</td>
<td>208.5 ± 32.0</td>
</tr>
<tr>
<td>IVSd (cm)</td>
<td>0.45 ± 0.08</td>
<td>0.44 ± 0.05</td>
<td>0.46 ± 0.10</td>
<td>0.56 ± 0.11</td>
<td>0.42 ± 0.04(^a)</td>
<td>0.52 ± 0.02(^b)</td>
<td>0.64 ± 0.10(^c)</td>
<td>0.42 ± 0.05</td>
<td>0.41 ± 0.04</td>
</tr>
<tr>
<td>LVWd (cm)</td>
<td>0.44 ± 0.09</td>
<td>0.42 ± 0.05</td>
<td>0.44 ± 0.10</td>
<td>0.57 ± 0.12</td>
<td>0.40 ± 0.04(^a)</td>
<td>0.51 ± 0.06(^b)</td>
<td>0.63 ± 0.11(^c)</td>
<td>0.41 ± 0.04</td>
<td>0.40 ± 0.04</td>
</tr>
<tr>
<td>Lvd (cm)</td>
<td>1.69 ± 0.21</td>
<td>1.72 ± 0.22</td>
<td>1.68 ± 0.21</td>
<td>1.57 ± 0.08</td>
<td>1.68 ± 0.22</td>
<td>1.75 ± 0.22</td>
<td>1.69 ± 0.13</td>
<td>1.71 ± 0.23</td>
<td>1.65 ± 0.22</td>
</tr>
<tr>
<td>LVs (cm)</td>
<td>0.80 ± 0.14</td>
<td>0.81 ± 0.14</td>
<td>0.80 ± 0.14</td>
<td>0.66 ± 0.11</td>
<td>0.81 ± 0.15</td>
<td>0.76 ± 0.14</td>
<td>0.78 ± 0.06</td>
<td>0.81 ± 0.15</td>
<td>0.80 ± 0.16</td>
</tr>
<tr>
<td>FS (%)</td>
<td>53.05 ± 4.91</td>
<td>52.94 ± 5.05</td>
<td>52.58 ± 4.57</td>
<td>58.11 ± 5.31</td>
<td>52.44 ± 4.79</td>
<td>56.41 ± 5.75</td>
<td>53.78 ± 3.32</td>
<td>52.72 ± 4.96</td>
<td>51.91 ± 4.71</td>
</tr>
<tr>
<td>Ao (cm)</td>
<td>0.96 ± 0.13</td>
<td>0.94 ± 0.12</td>
<td>0.97 ± 0.14</td>
<td>0.95 ± 0.18</td>
<td>0.95 ± 0.13</td>
<td>0.91 ± 0.13</td>
<td>1.08 ± 0.07</td>
<td>0.95 ± 0.12</td>
<td>0.95 ± 0.15</td>
</tr>
<tr>
<td>LA (cm)</td>
<td>1.19 ± 0.20</td>
<td>1.13 ± 0.16</td>
<td>1.23 ± 0.21</td>
<td>1.44 ± 0.28</td>
<td>1.16 ± 0.18(^a)</td>
<td>1.25 ± 0.19(^a)</td>
<td>1.40 ± 0.29(^c)</td>
<td>1.13 ± 0.16</td>
<td>1.20 ± 0.20</td>
</tr>
<tr>
<td>LA/Ao</td>
<td>1.25 ± 0.17</td>
<td>1.20 ± 0.14</td>
<td>1.27 ± 0.16</td>
<td>1.54 ± 0.33</td>
<td>1.23 ± 0.13</td>
<td>1.41 ± 0.30</td>
<td>1.29 ± 0.24</td>
<td>1.20 ± 0.12</td>
<td>1.26 ± 0.14</td>
</tr>
<tr>
<td>R Ao — Vmax</td>
<td>1.20 ± 0.71</td>
<td>0.99 ± 0.19(^a)</td>
<td>1.30 ± 0.90(^b)</td>
<td>2.23 ± 0.82</td>
<td>1.06 ± 0.23(^a)</td>
<td>1.14 ± 0.49(^b)</td>
<td>2.58 ± 1.85(^c)</td>
<td>1.00 ± 0.19</td>
<td>1.11 ± 0.23</td>
</tr>
<tr>
<td>R PA — Vmax</td>
<td>1.03 ± 0.16</td>
<td>1.01 ± 0.13</td>
<td>1.04 ± 0.19</td>
<td>1.05 ± 0.19</td>
<td>1.04 ± 0.16</td>
<td>0.99 ± 0.17</td>
<td>0.96 ± 0.14</td>
<td>1.03 ± 0.14</td>
<td>1.07 ± 0.20</td>
</tr>
<tr>
<td>E wave (m/s)</td>
<td>0.92 ± 0.21</td>
<td>0.90 ± 0.18</td>
<td>0.93 ± 0.21</td>
<td>1.03 ± 0.40</td>
<td>0.93 ± 0.20</td>
<td>0.87 ± 0.15</td>
<td>0.93 ± 0.38</td>
<td>0.92 ± 0.19</td>
<td>0.92 ± 0.21</td>
</tr>
<tr>
<td>A wave (m/s)</td>
<td>0.61 ± 0.15</td>
<td>0.61 ± 0.15</td>
<td>0.60 ± 0.13</td>
<td>0.85 ± 0.00</td>
<td>0.59 ± 0.15</td>
<td>0.63 ± 0.14</td>
<td>0.75 ± 0.10</td>
<td>0.60 ± 0.16</td>
<td>0.58 ± 0.14</td>
</tr>
<tr>
<td>E/A</td>
<td>1.33 ± 0.29</td>
<td>1.36 ± 0.27</td>
<td>1.35 ± 0.27</td>
<td>0.68 ± 0.00</td>
<td>1.38 ± 0.27</td>
<td>1.28 ± 0.35</td>
<td>1.03 ± 0.30</td>
<td>1.39 ± 0.25</td>
<td>1.36 ± 0.30</td>
</tr>
<tr>
<td>IVRT (ms)</td>
<td>55.68 ± 9.21</td>
<td>53.67 ± 5.93</td>
<td>56.38 ± 9.93</td>
<td>68.60 ± 19.0</td>
<td>54.09 ± 7.4(^a)</td>
<td>56.99 ± 7.8(^b)</td>
<td>71.1 ± 16.2(^c)</td>
<td>53.45 ± 5.60</td>
<td>54.74 ± 9.51</td>
</tr>
</tbody>
</table>

HCM — hypertrophic cardiomyopathy; HR — heart rate; IVSd — interventricular septum in diastole; LVWd — left ventricular wall in diastole; LVD — left ventricle in diastole; LVs — left ventricle in systole; FS — fractional shortening; Ao — aorta; LA — left atrium; LA/Ao — left atrium aorta ratio; Fl Ao — aortic flow; Vmax — maximum velocity; Fl PA — pulmonary artery flow; IVRT — isovolumetric relaxation time; E/A = E wave A wave ratio.

\(^a,b,c\) Statistical differences.
which may be explained by the racial pattern of the animals, based
although there have been reports of earlier and more evident clinical
elderly cats. Some studies have not reported gender differences,
male animals, although it may also occur in female and young or
(2,3,36), HCM has been more commonly reported in middle-aged
groups. However, the prevalence of male cats between the suspect
(5,6), which may hinder the interpretation and conclusions of the
known to be a genetically and phenotypically heterogeneous disease
related to HCM in humans (12,18,22), the absence of this mutation
apparently now also in cats, which makes it more difficult to diag-
nostic hereditable disorder in Maine coon cats. Incomplete penetrance
According to previous studies (32–35), HCM is an autosomal domi-
the existence of heterozygous and homozygous cats in this group.
Mean weight was higher for male animals than female animals,
(la) was greater in cats affected by HCM than in normal cats.
Differences in IVRT and velocity of aortic flow were noted and were
developed ventricular hypertrophy, which is consistent with what
masked the values obtained in this study. When evaluating only the
phenotypic alterations related to HCM, however, which may have
masked the values obtained in this study. When evaluating only the
group of normal cats had lower body weights than the other
groups (suspects and cats with HCM), there were more female ani-
imals in the normal group, which explains these findings.
The velocity of aortic flow was greater in cats heterozygous for the
mutation than in cats without the mutation. These cats also showed
phenotypic alterations related to HCM, however, which may have
masked the values obtained in this study. When evaluating only the
group without HCM, no significant differences were found regarding
genotype. Therefore, conventional echocardiography was unable to
differentiate cats without the mutation from those with it before they
developed ventricular hypertrophy, which is consistent with what
has been reported in the literature (2,4,37,38).
As for phenotypic classification, the diameter of the left atrium
(LA) was greater in cats affected by HCM than in normal cats.
Differences in IVRT and velocity of aortic flow were noted and were
greater in affected animals than in suspect and normal animals.
Three of the 5 cats with HCM had SAM, with increase in aortic flow.
When considering strain mode, regarding phenotypic evaluation,
significant differences were observed at the middle region of the
interventricular septum (IVS), with smaller values in cats with HCM
than in normal cats. Strain mode can be used for early quantification
of the degree of systolic dysfunction in certain stages of feline HCM
(20). In a study conducted with normal cats and cats in different
stages of HCM, although conventional echocardiography demon-
strated a normal condition or increased contraction (normal shortening
fraction), strain was lower in all animals with hypertrophy (20).

1 homozygote). Animals suspect for HCM accounted for 12% of the
evaluated population. Among these animals, 4 had the mutation
and 3 were negative. Most animals were normal (79%), despite
the existence of heterozygous and homozygous cats in this group.
According to previous studies (32–35), HCM is an autosomal domi-
nant hereditable disorder in Maine coon cats. Incomplete penetrance
is very common with this type of mutation in humans, however, and
apparently now also in cats, which makes it more difficult to diag-
ose heterozygotes through echocardiography. Moreover, as there
are over 1000 mutations in 10 genes that encode sarcomere proteins
related to HCM in humans (12,18,22), the absence of this mutation
does not exclude the possibility of the existence of other mutations
that may cause cardiomyopathy. Hypertrophic cardiomyopathy is
known to be a genetically and phenotypically heterogeneous disease
(5,6), which may hinder the interpretation and conclusions of the
present study.
Male and female distribution was homogeneous in the studied
groups. However, the prevalence of male cats between the suspect
and affected animals was significantly higher (60% of cats with HCM
and 71% of suspect cats were male). According to some authors
(2,3,36), HCM has been more commonly reported in middle-aged
male animals, although it may also occur in female and young or
elderly cats. Some studies have not reported gender differences,
although there have been reports of earlier and more evident clinical
manifestations in male animals (11).
Mean weight was higher for male animals than female animals,
which may be explained by the racial pattern of the animals, based
on which male cats are noticeably larger than female cats. Although
the group of normal cats had lower body weights than the other
groups (suspects and cats with HCM), there were more female ani-

### Table III. Mean and standard deviation of strain values in Maine coon cats according to genotype

<table>
<thead>
<tr>
<th>Parameters</th>
<th>All (n = 57)</th>
<th>No mutation (n = 28)</th>
<th>Heterozygous for the mutation (n = 26)</th>
<th>Homozygous for the mutation (n = 3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>4C-SI S (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HR</td>
<td>195 ± 27</td>
<td>192 ± 23</td>
<td>198 ± 33</td>
<td>194 ± 7</td>
</tr>
<tr>
<td>4CWB</td>
<td>−11.75 ± 3.71</td>
<td>−11.08 ± 3.54</td>
<td>−12.56 ± 3.98</td>
<td>−11.06 ± 1.23</td>
</tr>
<tr>
<td>4CWM</td>
<td>−12.44 ± 4.46</td>
<td>−13.08 ± 4.49</td>
<td>−12.25 ± 4.29</td>
<td>−8.02 ± 4.43</td>
</tr>
<tr>
<td>4CSB</td>
<td>−21.54 ± 6.52</td>
<td>−22.48 ± 5.52</td>
<td>−21.20 ± 7.18</td>
<td>−15.77 ± 7.98</td>
</tr>
<tr>
<td>4CSM</td>
<td>−23.19 ± 6.86</td>
<td>−24.44 ± 6.68</td>
<td>−23.22 ± 5.72</td>
<td>−11.30 ± 8.51</td>
</tr>
<tr>
<td>2C-SI S (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HR</td>
<td>194 ± 26</td>
<td>189 ± 21</td>
<td>199 ± 31</td>
<td>190 ± 18</td>
</tr>
<tr>
<td>2CPB</td>
<td>−13.27 ± 4.69</td>
<td>−13.81 ± 5.13</td>
<td>−12.53 ± 4.07</td>
<td>−14.48 ± 6.11</td>
</tr>
<tr>
<td>2CPM</td>
<td>−14.22 ± 5.93</td>
<td>−13.77 ± 5.85</td>
<td>−14.42 ± 5.55</td>
<td>−16.83 ± 11.11</td>
</tr>
<tr>
<td>2CAB</td>
<td>−16.69 ± 5.66</td>
<td>−18.82 ± 6.29</td>
<td>−14.58 ± 3.81</td>
<td>−14.41 ± 6.74</td>
</tr>
<tr>
<td>2CAM</td>
<td>−18.91 ± 5.65</td>
<td>−18.46 ± 5.53</td>
<td>−19.08 ± 5.08</td>
<td>−21.72 ± 11.86</td>
</tr>
</tbody>
</table>

Papillary
| HR            | 194 ± 28    | 192 ± 29             | 196 ± 30                              | 201 ± 8                           |
| SIS (%)        | 22.42 ± 7.19| 20.83 ± 6.44         | 24.50 ± 7.71                          | 19.92 ± 7.13                      |

HR — heart rate in beats per min; 4CWB — base of the left ventricular wall (LVW) at apical 4-chamber view; 4CWM — middle region of the LVW at apical 4-chamber view; 4CSB — base of the interventricular septum (IVS) at apical 4-chamber view; 4CSM — middle region of the IVS at apical 4-chamber view; 2CPB — base of the posterior left ventricular wall (PLW) at apical 2-chamber view; 2CPM — middle region of the PLW at apical 2-chamber view; 2CAB — base of the anterior left ventricular wall (ALWW) at apical 2-chamber view; 2CAM — middle region of the ALWW at apical 2-chamber view; SIS — systolic strain wave.

*a,b Statistical differences (a and b are different).
In humans, patients with HCM have also shown lower septal strain values than normal individuals, especially in the middle region of the septum in relation to the basilar region (19), which is similar to the results obtained in this study.

Regarding genotypic evaluation, a statistically significant difference was found between strain values obtained at the base of the ALVW, with lower values for heterozygotes than for negative animals. According to the literature, strain enables the direct quantification of the mutation in association with pathological remodeling (18). As no differences were found between carriers of the mutation (without hypertrophy) and normal patients, it can be concluded that, despite diastolic dysfunction secondary to sarcomere alterations, in early stages systolic function remains preserved. In another study conducted in humans who were carriers of a mutation that causes HCM and who had no hypertrophy (39), however, peak systolic strain assessed by echocardiography and magnetic resonance imaging was decreased before the presence of hypertrophy, which is similar to the results obtained herein.

In the present study, signs of regional systolic dysfunction were observed in cats heterozygous for the A31P mutation before ventricular hypertrophy had developed. On clinical evaluation, hypertrophic cardiomyopathy (HCM) is considered a disorder with predominantly diastolic dysfunction constituting changes in relaxation in the early stages and changes in ventricular distensibility in advanced stages (36). In humans, systolic and diastolic changes can be found on tissue Doppler echocardiography in patients with HCM. Diastolic dysfunction is a consequence of the mutation on the sarcomere, while systolic dysfunction is thought to result from the presence of the mutation in association with pathological remodeling (18).

Echocardiographic indices that assess systolic function are overestimated in the presence of ventricular hypertrophy and are dependent on preload and afterload conditions. Therefore, the...
Table V. Mean and standard deviation of strain values in negative and heterozygous phenotypically normal Maine coon cats

<table>
<thead>
<tr>
<th>Parameters</th>
<th>No HCM (n = 5)</th>
<th>No HCM and no mutation (n = 25)</th>
<th>Heterozygous for the mutation (n = 19)</th>
</tr>
</thead>
<tbody>
<tr>
<td>4C-StS (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HR</td>
<td>199 ± 28</td>
<td>194 ± 23</td>
<td>206 ± 34</td>
</tr>
<tr>
<td>4CWB</td>
<td>−11.80 ± 3.62</td>
<td>−11.44 ± 3.48</td>
<td>−12.32 ± 3.91</td>
</tr>
<tr>
<td>4CWM</td>
<td>−13.10 ± 4.44</td>
<td>−13.44 ± 4.56</td>
<td>−12.64 ± 4.49</td>
</tr>
<tr>
<td>4CSB</td>
<td>−22.14 ± 6.46</td>
<td>−22.32 ± 5.55</td>
<td>−21.76 ± 7.76</td>
</tr>
<tr>
<td>4CSM</td>
<td>−24.27 ± 6.34</td>
<td>−24.45 ± 6.83</td>
<td>−24.20 ± 5.95</td>
</tr>
<tr>
<td>2C-StS (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HR</td>
<td>196 ± 28</td>
<td>190 ± 22</td>
<td>206 ± 33</td>
</tr>
<tr>
<td>2CPB</td>
<td>−13.33 ± 4.89</td>
<td>−13.7 ± 5.30</td>
<td>−13.16 ± 4.30</td>
</tr>
<tr>
<td>2CPM</td>
<td>−14.11 ± 5.98</td>
<td>−13.74 ± 6.20</td>
<td>−14.49 ± 5.95</td>
</tr>
<tr>
<td>2CAB</td>
<td>−17.06 ± 5.82</td>
<td>−18.79 ± 6.47(^a)</td>
<td>−14.59 ± 3.98(^b)</td>
</tr>
<tr>
<td>2CAM</td>
<td>−19.60 ± 5.70</td>
<td>−18.71 ± 5.75</td>
<td>−20.09 ± 4.83</td>
</tr>
</tbody>
</table>

Papillary

<table>
<thead>
<tr>
<th>Parameters</th>
<th>No HCM (n = 5)</th>
<th>No HCM and no mutation (n = 25)</th>
<th>Heterozygous for the mutation (n = 19)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HR</td>
<td>199 ± 28</td>
<td>196 ± 28</td>
<td>203 ± 27</td>
</tr>
<tr>
<td>StS (%)</td>
<td>22.08 ± 6.89</td>
<td>21.0 ± 6.23</td>
<td>23.25 ± 7.76</td>
</tr>
</tbody>
</table>

HCM — hypertrophic cardiomyopathy; HR — heart rate in beats per min; 4CWB — base of the left ventricular wall (LVW) at apical 4-chamber view; 4CWM — middle region of the LVW at apical 4-chamber view; 4CSB — base of the interventricular septum (IVS) at apical 4-chamber view; 4CSM — middle region of the IVS at apical 4-chamber view; 2CPB — base of the posterior left ventricular wall (PLVW) at apical 2-chamber view; 2CPM — middle region of the PLVW at apical 2-chamber view; 2CAB — base of the anterior left ventricular wall (ALVW) at apical 2-chamber view; 2CAM — middle region of the ALVW at apical 2-chamber view; StS — systolic strain wave.

\(^a\) Statistical differences.

The main limitations of this study are the small number of animals evaluated and the small number of homozygotes, which prevented statistical comparisons, thus reducing the chance of detecting early changes in these animals. Another limitation relates to the young age of the population. Although most cats were within the age range for the highest occurrence of HCM, 10% of the studied population were younger than 1.5 y. Therefore, the possibility of finding more evident diastolic dysfunctions, or even HCM, if these animals had been evaluated at older ages cannot be excluded. Another limitation is the lack of validation to quantify the systolic longitudinal LV motion assessed from the apical 2-chamber view. More studies of the strain imaging technique in the 2-chamber longitudinal view must be carried out in order to validate this method with high repeatability and reproducibility.

In many cats, tachycardia resulted in impaired echocardiographic evaluation and interpretation. The fusion of E and A waves was common on the different sections, which impaired the analyses.

In conclusion, strain imaging is a new echocardiographic mode that detects systolic abnormalities in Maine coon cats with mutations in the MYBPC3 gene, despite the apparently normal to increased shortening fraction (contraction) on conventional echocardiography. Cats with HCM and without clinical signs presented some alterations in indices of systolic function obtained through strain. Strain echocardiography could be a useful tool for detecting systolic alterations in HCM cats with an apparently normal systolic function or for detecting alterations in normal carriers of the MYBPC3 gene mutation.

Acknowledgments

The authors thank the State of São Paulo Research Foundation (FAPESP) and the Brazilian Association of Maine Coon Breeders (AMACOON) for supporting this study.

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Prokaryotic expression of the extracellular domain of porcine programmed death 1 (PD-1) and its ligand PD-L1 and identification of the binding with peripheral blood mononuclear cells *in vitro*

Yan-Ping Zhu, Feng Yue, Yong He, Peng Li, Yuan Yang, Yu-Ting Han, Yan-Fang Zhang, Guo-Peng Sun, Dong-Guang Guo, Mei Yin, Xuan-Nian Wang

**Abstract**

Programmed cell death protein 1 (PD-1), a costimulatory molecule of the CD28 family, has 2 ligands, PD-L1 and PD-L2. Our previous studies showed that the expression of PD-1 and PD-L1 is up-regulated during viral infection in pigs. Extensive studies have shown that blockade of the PD-1/PD-L1 pathways by anti-PD-L1 antibody or soluble PD-1 restores exhausted T-cells in humans and mice. In the present study the extracellular domains of PD-1 and PD-L1 were used to evaluate the binding of PD-1 and PD-L1 with peripheral blood mononuclear cells (PBMCs). We amplified the cDNA encoding the extracellular domains of PD-1 and PD-L1 to construct recombinant expression plasmids and obtain soluble recombinant proteins, which were then labeled with fluorescein isothiocyanate (FITC). The His-ExPD-1 and His-ExPD-L1 recombinant proteins were expressed in the form of inclusion bodies with a relative molecular weight of 33.0 and 45.0 kDa, respectively. We then prepared polyclonal antibodies against the proteins with a multi-antisera titer of 1:102,400. Binding of the proteins with PBMCs was evaluated by flow cytometry. The fluorescence signals of His-ExPD-1-FITC and His-ExPD-L1-FITC were greater than those for the FITC control. These results suggest that the soluble recombinant proteins may be used to prepare monoclonal antibodies to block the PD-1/PD-L1 pathway.

**Résumé**


**Introduction**

Optimal activation of T-cells for clonal expansion depends on 2 distinct signals from antigen-presenting cells. One is the delivery of specific antigen to the T-cell receptor by specific peptides in the context of major histocompatibility proteins on antigen-presenting cells; the other is triggered through a distinct T-cell surface molecule. The immunoglobulin (Ig) B7-CD28 superfamily, one of the best-characterized costimulatory receptor families, not only provides critical positive second signals to initiate and sustain the T-cell response but also contributes key negative second signals to down-regulate and terminate the T-cell response (1–4). The negative second signals include many cell surface molecules, especially negative receptors, such as cytolytic T-lymphocyte-associated Ag-4 (CTLA-4) (5), programmed cell death protein 1 (PD-1) (6), T-cell immunoglobulin and mucin-domain-containing molecule 3 (Tim-3) (7), lymphocyte-activation protein 3 (LAG-3) (8), and forkhead box P3 (FoxP3) (9).

Among these negative receptors, PD-1 plays an important role in reversible immune dysfunction (10). It is a 55.0-kDa type I transmembrane glycoprotein of the CD28 superfamily. Its single extracellular Ig variable (V)-like domain (11–13) is expressed on activated...
T-cells, B-cells, and monocytes (14–16). Porcine PD-1 has 63% and 54% identity with the human and murine PD-1, respectively, and a similar structure, with 2 highly hydrophobic amino acid fragments constituting the signal peptide (amino acids 1 to 20) and the transmembrane domain (amino acids 168 to 194), as well as an extracellular domain and a cytoplasmic domain (17,18). The extracellular domain plays an important role in the binding of PD-1 to its 2 known ligands, PD-L1 (19,20) and PD-L2 (21,22). Many studies have demonstrated that a high level of PD-1 expression is closely related to infection in humans with viruses such as human immunodeficiency virus, hepatitis B virus, and hepatitis C virus (23–25). Binding of PD-1 with its 2 ligands inhibits the proliferation of T-cells and the production of cytokines, especially interleukin-2 and interferon gamma (20, 21). With blockade of the interaction between PD-1 and PD-L1 by antibodies or soluble proteins, impaired T-cells can regain their ability to proliferate, secrete cytokines, and kill infected cells (6,26–34). Our previous research demonstrated that the expression of PD-1 and PD-L1 is up-regulated during viral infection in pigs (35). In the present study the extracellular domains of PD-1 and PD-L1 were used to evaluate the function of PD-1 and PD-L1 during viral infection in pigs. Recombinant protein obtained by high-level expression and purification interacted in vitro with peripheral blood mononuclear cells (PBMCs) from pigs infected with classical swine fever virus (CSFV).

Materials and methods

Cloning of extracellular domains

Primers were designed according to the porcine PD-1 and PD-L1 gene sequences (NM_001204379.1 and NM_001025221.1) published in GenBank (National Center for Biotechnology Information, Bethesda, Maryland, USA). Important PD-1 and PD-L1 gene sites were modified according to an analysis of codon bias of Escherichia coli (36), and the integrated genes were synthesized by Shanghai Bio-engineering Company, Shanghai, China. The regions encoding the extracellular domains were then amplified by polymerase chain reaction (PCR). Total RNA was extracted from PBMCs with the use of Trizol reagent (Invitrogen, Carlsbad, California, USA) to an optical density at 600 nm (OD600) of 0.5 to 1.0, induced with 0.5 mmol/L of isopropylthiogalactosidase (IPTG) for 4 h, and centrifuged to collect cell supernatant and cellular pellets for further analyses.

Identification of His-ExPD-1 and His-ExPD-L1 recombinant proteins

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot testing were done according to the manufacturers’ protocols (38). Briefly, 100 µL of cell supernatant or cellular pellets were resuspended in equal volumes of SDS-loading buffer. After electrophoresis the gel was stained with Coomassie Brilliant Blue R-250 (Sigma-Aldrich, St. Louis, Missouri, USA). Nitrocellulose membrane (Bio-Rad Laboratories, Hercules, California, USA) for the Western blot testing was blocked overnight with Tris-buffered saline (TBS; 20 mM Tris-HCl, pH 7.4, and 150 mM NaCl) containing 5% (w/v) nonfat dried milk, rinsed 3 times, and then immersed overnight in 20 mL of TBS containing 1% (w/v) nonfat dried milk, 1 µL of 6 × His-tag mouse monoclonal antibodies (PBL Biomedical Laboratories, Piscataway, New Jersey, USA), and 1 µL of mouse anti-human PD-1 monoclonal antibodies (Abcam, Cambridge, Massachusetts, USA) as the primary antibodies. Subsequently the membrane was rinsed 3 times and then immersed overnight in 20 mL of TBS containing 1% (w/v) nonfat dried milk and 1 µL of goat IgG monoclonal antibodies labeled with horseradish peroxidase (PBL) against mouse IgG as the secondary antibody. Then the membrane was rinsed 3 times and stained with dianinobenzidine (Sigma-Aldrich) to visualize peroxidase activity.

Protein purification and measurement

The greatest expression of the recombinant proteins was induced by IPTG under optimized conditions. Cells were harvested, the supernatant was discarded, and the inclusion body pellet was resuspended in phosphate buffer and centrifuged for 20 min. The cell pellets were then solubilized in 10 mL of elution buffer containing urea (0.1 mol/L of NaH2PO4·H2O, 0.01 mol/L of Tris base, 8 mol/L of urea, and 20 mmol/L of imidazole, adjusted to a pH of 8.0 with concentrated HCl). The precipitate was resuspended by centrifugation. The supernatant was collected and filtered with a 0.4-µm membrane and then loaded on a manually packed column containing 2 mL of nickel-nitrilotriacetic acid–agarose resin according to the instructions of the manufacturer (Qiagen, Valencia, California, USA).

Bovine serum albumin was diluted into 8, 4, 2, 1, 0.5, 0.25, and 0.125 mg/mL solutions and the OD280 of the different concentrations determined to establish a standard curve. With the same method the OD280 of His-ExPD-1 and His-ExPD-L1 was also determined and calculated according to the standard curve.
Preparation of polyclonal antibodies to the recombinant proteins

Four New Zealand female specific-pathogen-free rabbits (body mass 2 to 3 kg) were purchased from the Laboratory Animal Center, Henan Academy of Agricultural Sciences, Zhengzhou, Henan Province, China, and maintained under conventional conditions with food and water provided ad libitum. All experimental procedures were conducted according to institutional guidelines for animal ethics. On day 0, negative-control blood samples were collected and the rabbits vaccinated as previously described (39). Briefly, the rabbits were divided into 2 groups and injected intramuscularly with either His-ExPD-1 or His-ExPD-L1, 500 mg emulsified in 500 µL of phosphate-buffered saline (PBS)/Freund’s complete adjuvant. Boosters were given on days 15, 29, and 44 with the same dose and by the same route of antigen in Freund’s incomplete adjuvant. On day 54, blood samples were collected to be tested by Western blot or enzyme-linked immunosorbent assay (ELISA) for antibody activity. The ELISA results were expressed as OD_{630} and OD_{450} for each sample. Mean values for the positive (mouse anti-Human PD-1 and mouse anti-6His monoclonal antibodies) and negative controls were obtained as the average value for 2 wells. The sample/positive (S/P) value was calculated with the following formula: \( S/P = \frac{OD_{450} + OD_{630}}{OD_{450} + OD_{630}} \) of negative control. Samples with an S/P value of less than 3 were classified as negative and samples with an S/P value of 3 or greater as positive. When the antibody levels peaked, the rabbits were killed to collect blood. Cell-free serum was decanted gently into a clean test tube and stored at \(-20°C\) for further study.

Protein labeling

The amino acid residues imidazole, carbonyl, and cheese ammonia acyl groups of the His-ExPD-1 and His-ExPD-L1 proteins were labeled with fluorescein isothiocyanate (FITC) by means of an antibody labeling kit (Applied Biosystems, Foster City, California, USA) according to the manufacturer’s protocol, as follows. First, 40 µL of the borate buffer (0.67 M) was added to 0.5 mL of protein in PBS (2 mg/mL). Then 0.5 mL of the prepared protein was added to the vial of FITC reagent (30 µL) and pipetted up and down 10 times until all the dye was dissolved. The vial was briefly centrifuged to collect the sample in the bottom of the tube. The reaction mixture was incubated for 60 min at room temperature, protected from light. Second, 2 spin columns were placed in separate microcentrifuge collection tubes. The purification resin was mixed to ensure uniform suspension, and 400 µL of the suspension was added to both spin columns. The stored solution was centrifuged for 30 to 45 s at about \(1000 \times g\) to remove the storage solution. The used collection tubes were discarded and the columns placed in new collection tubes. Third, 250 to 270 µL of the labeled reaction mixture was added to each spin column and mixed with the resin by pipetting up and down or briefly vortexing. The columns were centrifuged for 30 to 45 s at about \(1000 \times g\) to collect the purified proteins. Alternatively, labeled proteins were stored in single-use aliquots at \(-20°C\).

Identification of protein binding with PBMCs in vitro

We isolated PBMCs from 45-day-old pigs weighing approximately 15 kg that were provided by pig breeding center of Henan province,
(Zhengzhou, Henan Province, China) for our previous study (35). All experimental procedures were conducted according to institutional guidelines for animal ethics. In the previous study we had found the expression levels of PD-1 and PD-L1 mRNA to be significantly up-regulated in the pigs experimentally infected with CSFV compared with the control pigs at 3 d ($P < 0.05$) and 7 d ($P < 0.01$) after infection. The PBMCs were diluted with PBS to $1 \times 10^6$ cells/mL and divided into 2 experimental groups and a control group. They were then resuspended in $100 \mu$L of phosphate buffer and incubated for 30 min at 4°C in a mixed solution of $10 \mu$L/mL of His-ExPD-1-FITC, His-ExPD-L1-FITC, or FITC as the control. Washing buffer (PBS, $1 \text{ mL}$) was added to collect the PBMCs, which were then resuspended in $1 \text{ mL}$ of phosphate buffer and filtered through a 200-μm mesh screen. Flow cytometry was used to recognize PD-1 and PD-L1 on the surface of the PBMCs.

Results

After cloning of the ExPD-1 and ExPD-L1 gene regions encoding the extracellular domains of PD-1 and PD-L1, DNA fragments of

Figure 2. Results of His-ExPD-1 analysis by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE) (A) and Western blot testing with, as primary antibodies, $6 \times$ His-tag mouse monoclonal antibodies (B) and mouse PD-1 monoclonal antibodies against human antigen (C). A, lane M — low molecular weight standard prestained marker (Fermentas, Waltham, Massachusetts, USA); lanes 1 to 3 — recombinant Rosetta (DE3) cells transformed with pET-32a-PD-1, uninduced; lanes 4 to 6 — recombinant Rosetta (DE3) cells transformed with pET-32a-PD-1 and induced by isopropylthiogalactosidase (IPTG); lane 7 — recombinant Rosetta (DE3) cells electrotransformed with pET-32a(+) and induced by IPTG; lane 8 — Rosetta (DE3) cells induced by IPTG as a negative control. B and C, lane M — low molecular weight standard prestained marker; lane 1 — recombinant Rosetta (DE3) cells transformed with pET-32a-PD-1 and induced by IPTG; lane 2 — recombinant Rosetta (DE3) cells transformed with pET-32a-PD-1, uninduced; lane 3 — recombinant Rosetta (DE3) cells electrotransformed with pET-32a(+) and induced by IPTG; lane 4 — Rosetta (DE3) cells induced by IPTG as a negative control. A gel band revealed the expressed protein to be about 33.0 kDa, and Western blot testing with goat monoclonal antibodies to mouse IgG labeled with horseradish peroxidase as the secondary antibody confirmed that the 33.0-kDa band was the His-ExPD-1 protein (B and C).
366 base pairs (bp) and 684 bp were obtained by PCR (Figure 1), double enzymatic digestion, and DNA sequencing. High-level production of recombinant protein in bacteria was induced by IPTG under optimized conditions: these proteins, His-ExPD-1 and His-ExPD-L1, were in the form of inclusion bodies with relative molecular weights of 33.0 and 45.0 kDa, which agreed with the predicted values from the gene sequences (Figures 2A and 3A).

Western blot testing indicated that the recombinant proteins were recognized by 6 × His-tag mouse monoclonal antibodies (Figures 2B and 3B); His-ExPD-1 was also recognized by PD-1 mouse monoclonal antibodies against human antigen (Figure 2C), which showed that human PD-1 and porcine His-ExPD-1 recombinant protein have high protein homology, not only high sequence homology. The SDS-PAGE and Western blot results suggested that the recombinant expression

Figure 3. Results of His-ExPD-L1 analysis by SDS-PAGE (A) and Western blot testing (B). A, lane M — low molecular weight standard prestained marker; lanes 1, 3, 5, and 7 — recombinant Rosetta (DE3) cells transformed with pET-32a-PD-L1 and induced by IPTG; lanes 2, 4, 6, and 8 — recombinant Rosetta (DE3) cells electrotransformed with pET-32a-PD-L1, uninduced; lane 9 — recombinant Rosetta (DE3) cells electrotransformed with pET-32a(+) and induced by IPTG; lane 10 — Rosetta (DE3) cells induced by IPTG as a negative control. B, lane M — low molecular weight standard prestained marker; lane 1 — recombinant Rosetta (DE3) cells transformed with pET-32a-PD-L1 and induced by IPTG; lane 2 — Rosetta (DE3) cells induced by IPTG as a negative control; lane 3 — recombinant Rosetta (DE3) cells electrotransformed with pET-32a(+) and induced by IPTG. A gel band revealed the expressed protein to be about 45.0 kDa, and Western blot testing confirmed that the 45.0-kDa band was the His-ExPD-L1 protein.

Figure 4. Results of analysis of the purity of the purified recombinant His-ExPD-1 (A) and His-ExPD-L1 (B) proteins. A, lane M — low molecular weight standard prestained marker; lane 1 — purified His-ExPD-1. B, lane M — low molecular weight standard prestained marker; lane 1 — purified His-ExPD-L1.
plasmid could effectively express His-ExPD-1 and His-ExPD-L1 as inclusion bodies.

Under optimum induction conditions the recombinant proteins were abundantly expressed, constituting as much as 95% of the total cell protein (Figure 4). The levels of His-ExPD-1 and His-ExPD-L1 were 0.9 and 1.5 mg/mL, respectively, as determined by protein scanning and ultraviolet absorbance.

The Western blot results suggested that polyclonal antibodies against His-ExPD-1 and His-ExPD-L1 recombinant proteins could react with PD-1 and PD-L1 recombinant proteins. The multiantiserum titters of His-ExPD-1 and His-ExPD-L1 were both 1:102 400.

The fluorescence signals of His-ExPD-1-FITC and His-ExPD-L1-FITC were greater than those of the FITC control (Figure 5). The flow cytometry results showed that His-ExPD-1-FITC and His-ExPD-L1-FITC could bind with PBMCs in vitro. These results suggest that these recombinant proteins have the biologic activity of the natural porcine PD-1 and PD-L1 proteins.

**Discussion**

Porcine PD-1 and its ligand PD-L1 are type I transmembrane glycoproteins of the B7-CD28 Ig receptor superfamily with a single IgV-like extracellular domain (11–13,40). The crystal structure of the PDI/PD-L2 complex suggested that PD-L1 and PD-L2 may form similar assemblies with PD-1 (41). The binding interfaces were formed by the front β-sheets of both the PD-1 IgV domain (labeled ABED and A’GFCC’ C’) and the PD-L2 IgV domain (labeled AGFCC’C’ and BED). Side chains of residues on β-strands (CC’FG) of PD-1 and on β-strands (GFCC’) of PD-L1 made PD-1/PD-L1 contacts interface (40). Residues from the GFCC’ strands and the CC’, C’, and FG loops of PD-1 contributed to the binding interface and packed against the AGFC strands and the FG loop of the PD-L2 IgV domain, as well as the AGFC strands and the CC’ loop of the PD-L1 IgV domain (40). These reports suggest that the extracellular domains of PD-1 and its ligands play an important role in their interactions. Therefore, antibodies and soluble proteins of the extracellular domain are potent inhibitors of PD-1 and PD-L1.

Previous studies showed that producing biologically active recombinant protein in bacteria was feasible (4). In this study, the extracellular domains of porcine PD-1 and PD-L1 were the object of study to obtain soluble protein. Residues of the PD-L1 V domain that bind to PD-1 are conserved across species (40). The residues (Ala-121, Asp-122, Tyr-123, and Lys-124 in the G strand of PD-L1) make intimate contacts with PD-1 and are conserved in all available PD-L1 sequences from mammals and birds (40). The extracellular domains of murine PD-1 and of human PD-L1 were from Leu-25 to Ser-157 and from Ala-18 to Thr-239, respectively (40). According to the published porcine PD-1 and PD-L1 gene sequences (NM_001204379.1 and NM_001025221.1) in GenBank, expression constructs encoding the extracellular domains of porcine PD-1 and PD-L1 were from Leu-24 to Leu-166 and from Val-23 to Thr-237, respectively.

The His-ExPD-1 and His-ExPD-L1 recombinant proteins were generated with use of the well-known polyhistidine tag (6 × His) fusion system, with the pET-32a(+) expression vector, which provided high-level expression and easy purification (42). The recombinant proteins were almost all in the form of inclusion bodies and soluble protein. Soluble active production can be induced by altering expression conditions (temperature, induction time, IPTG concentration, cell density aeration, or pH of culture). Unfortunately, all methods were unsuccessful at inducing soluble active products. Therefore, soluble biologically active protein was obtained by denaturing and refolding the inclusion bodies. Finally, high-quality protein with good bioactivity was obtained by optimizing the refolding conditions. Protein scanning and ultraviolet absorbance were used to elevate the purity, to more than 95%. Thus, this method can be used to obtain these proteins in high amounts and high purity for preparing polyclonal antibodies, monoclonal antibodies, and FITC-labeled proteins for further study.

Murine PD-1 can bind with human PD-L1 (40), which can interact with porcine PD-1 (17,43,44), and human PD-1 can bind with porcine PD-L1 (43). The conformations of PD-1 from different species are highly conserved. Thus, mouse PD-1 against human antigen was used herein as the primary antibody for Western blot testing with porcine His-ExPD-1 recombinant protein. The results hint that porcine PD-1 may play a negative role in immune regulation similar to that of human and murine PD-1. We will evaluate this hypothesis in future experiments.

Our previous analysis by quantitative real-time PCR of the biologic activity of His-ExPD-1 and His-ExPD-L1 with PBMCs from pigs with CSFV infection showed that the expression of PD-1 and PD-L1 mRNA was significantly upregulated compared with that in the control groups (35). Therefore, in the present study natural PD-1 and PD-L1 on the surface of PBMCs were used to evaluate the bioactivity of His-ExPD-1 and His-ExPD-L1. We found that His-ExPD-1-FITC and His-ExPD-L1-FITC could recognize natural PD-1 and PD-L1 proteins on PBMCs. The results suggest that His-ExPD-1 and His-ExPD-L1 recombinant proteins can bind with natural PD-1 and PD-L1 on the surface of PBMCs from pigs and therefore could be used to develop monoclonal antibodies that can block the PD-1/ PD-L1 pathway to restore immune status. Furthermore, with blockade of the interaction between PD-1 and PD-L1 by soluble proteins,
impaired T-cells are able to regain their ability to proliferate, secrete cytokines, and kill infected cells (6,26–34). Therefore, His-exPD-L1 and His-ExPD-L1 recombinant proteins, or even their polyclonal antibodies, could be used to block the PD-1/PD-L1 pathway in pigs during the virus infection. Other biologic characteristics of His-exPD-1 and His-ExPD-L1 and their possible roles during virus infection in pigs in vitro and in vivo are being investigated in our laboratory.

Acknowledgments

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References


Prevalence of small ruminant lentivirus and *Mycobacterium avium* subsp. *paratuberculosis* co-infection in Ontario dairy sheep and dairy goats

Nancy Stonos, Cathy Bauman, Paula Menzies, Sarah K. Wootton, Niel A. Karrow

**Abstract**

Infection with small ruminant lentiviruses (SRLV) causes a variety of chronic inflammatory conditions that limit production. *Mycobacterium avium* subsp. *paratuberculosis* (MAP) is also a major production-limiting disease of sheep and goats, which causes severe inflammation of the small intestine. Previous studies have indicated that both SRLV and MAP are widespread in small ruminants in Ontario. This study estimated the prevalence of SRLV and MAP co-infection. Serum samples that were previously tested for MAP infection were re-tested for SRLV. The apparent prevalence of co-infection was low, with 3.4% [95% confidence interval (CI): 1.9 to 5.9] and 14.3% (95% CI: 11.6 to 17.5) of sheep and goats respectively, positive for both infections. However, co-infection is widespread with 36.8% (95% CI: 19.1 to 59.1) and 71.4% (95% CI: 52.8 to 84.9) of sheep and goat farms with 1 or more co-infected animals. A significant association was found between SRLV seropositivity and MAP fecal culture ($P = 0.021$), suggesting that co-infected goats may be more likely to shed MAP in their feces.

**Résumé**

L’infection par lentivirus des petits ruminants (SRLV) provoque une variété d’états inflammatoires chroniques qui limitent la production. *Mycobacterium avium* subsp. *paratuberculosis* (MAP) est aussi une maladie limitant la production majeure de moutons et de chèvres, ce qui provoque une inflammation grave de l’intestin grêle. Des études antérieures ont indiqué que les deux infections de SRLV et MAP sont très répandues dans l’Ontario petits ruminants. Cette étude a été réalisée pour estimer la prévalence de SRLV et MAP co-infection. Des échantillons de sérum qui avaient été préalablement testés pour l’infection de MAP ont été utilisés pour détecter des anticorps spécifiques SRLV. La prévalence de la co-infection était faible, avec 3.4 % intervalle de confiance (95% IC : 1.9–5.9) et 14.3 % (95% IC : 11.6–17.5) des ovins et caprins, respectivement, positive pour les deux infections. Cependant la co-infection est très répandue avec 36.8 % (95% IC : 19.1–59.1) et 71.4 % (95% IC : 52.8–84.9) des élevages ovins et caprins avec un ou plusieurs animaux co-infecté. Une association significative a été trouvée entre SRLV et séropositivité MAP culture fécale ($P = 0.021$), ce qui suggère que les chèvres co-infectés peuvent être plus susceptibles de jeter le MAP dans leurs excréments.

The maedi-visna virus (MVV) and caprine arthritis encephalitis virus (CAEV) are lentiviruses affecting sheep and goats, respectively (1). The MVV and CAEV can infect either species and an individual animal can be infected with 1 or more strains; therefore, these viruses are often referred to as the small ruminant lentivirus (SRLV) (1). The SRLV infects monocytes and macrophages, and causes chronic inflammation in tissues, such as the lungs, mammary glands, central nervous system, and joint synovia (2). Common clinical signs include progressive weight loss, hard udders with reduced milk production, chronic respiratory disease in sheep, and enlarged joints with lameness in goats (2). However, due to the long incubation period of SRLV, it can take months or years after infection for clinical signs of disease to emerge.

Transmission of SRLV primarily occurs when the newborn lamb or kid is exposed to shed viron particles and infected cells in colostrum and milk, or through infected respiratory secretions from the dam or other near-by adults (2). However, SRLV can also be transmitted directly to the fetus in utero or during the birth process (2). Horizontal transmission can also occur among adult sheep through direct contact with infected respiratory secretions, and sexual transmission may also be possible (2).

Johne’s disease is a production-limiting disease of ruminants caused by infection with *Mycobacterium avium* subsp. *paratuberculosis* (MAP) (3). In small ruminants, the infection causes chronic inflammation of the distal ileum leading to progressive weight loss with or without diarrhea (3). As with SRLV, Johne’s disease has a long incubation period from 1 to several years (3). However, MAP bacteria can be shed in the feces for many months prior to the onset of clinical signs making control difficult (3). The MAP bacteria infect macrophages located in Peyer’s patches beneath the intestinal epithelium of the distal ileum. The bacteria replicate inside these cells forming granulomatous lesions (4). This localized infection allows MAP to
evade host immune responses while shedding bacteria into the feces and thus into the environment (4). As the infection progresses, monocytes and lymphocytes traffic to the infection site, lesions continue to grow, and, as the epithelial cell layer becomes increasingly damaged, clinical signs emerge (4). The primary mode of MAP transmission is fecal-oral from feces and the contaminated environment and skin, such as teats of the dam (5). The MAP can also be transmitted to newborn animals through bacterial shedding in the colostrum and milk, and transplacental infection may occur (5).

Previously, the apparent prevalence of SRLV infection in Ontario goats based on serology was 17% and 80.4% in meat and dairy goats, respectively (6), and 20.9% in Ontario sheep (7). Using fecal culture, the apparent prevalence of MAP infection and the individual animal level was estimated at 18.3% (95% CI: 12.0 to 24.6) and 7.6% (95% CI: 4.7 to 9.7) in dairy goats and dairy sheep, respectively, while herd-level apparent prevalence was 79.3% (95% CI: 60.3 to 90.2) and 57.1% (95% CI: 34.0 to 78.0) in dairy goat herds and dairy sheep flocks, respectively (8). Given the high prevalence of SRLV and the widespread nature of MAP infection, it is probable that herds and, likely, individual animals may be co-infected. Since both SRLV and MAP preferentially infect macrophages and monocytes, it is unclear how animals respond when faced with both pathogens. Additionally, both SRLV and MAP cause severe immune dysregulation as the disease progresses (9,10); therefore, understanding how these pathogens interact and affect host immunity is important for overall herd and flock health. The prevalence of SRLV and MAP co-infection, however, is currently unknown. A follow-up investigation using samples from a previous MAP study (8) was conducted to validate the prevalence.
Table I. A 2 × 2 representation of SRLV and MAP infection in lactating dairy sheep > 2 years of age (n = 355) from 19 Ontario sheep herds (P = 0.592)

<table>
<thead>
<tr>
<th></th>
<th>SRLV positive</th>
<th>SRLV negative</th>
<th>Total</th>
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<tbody>
<tr>
<td>MAP negative</td>
<td>134</td>
<td>197</td>
<td>331</td>
</tr>
<tr>
<td>MAP positive</td>
<td>12</td>
<td>12</td>
<td>24</td>
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<tr>
<td>Total</td>
<td>146</td>
<td>209</td>
<td>355</td>
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Table II. A 2 × 2 representation of SRLV and MAP infection in lactating dairy goats > 2 years of age (n = 558) from 28 Ontario goat herds (P = 0.0213)

<table>
<thead>
<tr>
<th></th>
<th>SRLV positive</th>
<th>SRLV negative</th>
<th>Total</th>
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<tbody>
<tr>
<td>MAP negative</td>
<td>354</td>
<td>102</td>
<td>456</td>
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<tr>
<td>MAP positive</td>
<td>93</td>
<td>9</td>
<td>102</td>
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<tr>
<td>Total</td>
<td>447</td>
<td>111</td>
<td>558</td>
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of SRLV in Ontario and estimate the prevalence of co-infection in Ontario dairy sheep flocks and dairy goat herds.

Serum samples from 355 sheep and 558 goats that were previously collected to evaluate diagnostic test accuracy for MAP infection (8) were then evaluated for evidence of SRLV infection, thus allowing for the determination of the prevalence of co-infection with MAP and SRLV. These samples were collected between October 2010 and August 2011 from 19 dairy sheep farms and 28 dairy goat farms in Ontario. Dairy goat herds were selected randomly after stratification based on herd size, while the sheep flocks were selected conveniently since a list of Ontario dairy sheep producers was unavailable (8). From each herd/flock, blood and feces were collected from 20 randomly selected, healthy-appearing lactating females > 2 y of age. The MAP and SRLV disease status of all herds and flocks was unknown at the time of sampling. Fecal samples were evaluated for presence of MAP bacteria by culture using a detection system (BD BACTEC MGIT 960 Mycobacterial detection system; Becton Dickinson and Company, Franklin Lakes, New Jersey, USA) and BACTEC MGIT Para TB medium confirmed by acid-fast staining and polymerase chain reaction (PCR) (Tetracore, Rockville, Maryland, USA) (8). A sample was confirmed fecal culture positive if it fluoresced positive in the BACTEC system, had a positive acid-fast stain and positive PCR confirmatory test.

The serum samples were subsequently submitted to Animal Health Laboratory, University of Guelph, Guelph, Ontario to test for the presence of antibodies to SRLV. Sheep sera were tested using the Hyphen ELISA test (ELITEST MVV/CAEV; Hyphen Biomed, Neuville sur Oise, France), and goat sera was tested using an enzyme-linked immunosorbent assay (ELISA) kit (CAEV ELISA kit, IDEXX CHEKIT/MVV, IDEXX Laboratories, Westbrook, Maine, USA) according to the manufacturer’s instructions. The standard protocols for both tests were previously described by DeAndres et al (11) and Stonos et al (6), respectively.

For the purposes of this study, an animal was considered positive for MAP infection if positive by fecal culture. This test was chosen because it is more sensitive than serology (8). An animal was considered positive for SRLV infection if the ELISA result was positive. A farm was considered MAP or SRLV positive if ≥ 1 animal in the herd/flock tested positive for either. An animal is defined as co-infected if positive for both SRLV serology and MAP fecal culture.

The data were organized and stored using computer software (Microsoft Excel version 14.4.8 Redmond, Washington, USA). Sheep and goat data were analyzed separately due to species-specific differences in test performance (12). The SRLV prevalence data were analyzed using the computer software (GLIMMIX, SAS Version 9.2; SAS Institute, Cary, North Carolina, USA). This procedure used a generalized linear model with a logit-link function \( \log(p(\text{infected}))/\end{align*} and included farm as a random effect with MAP serum results, MAP fecal culture results, and MAP polymerase chain reaction (PCR) results as fixed effects. This model allowed for the inclusion of SRLV infection as a binary trait. Further analysis with the mixed model involved the logistical regression analysis using computer software (SAS). This model used log transformed SRLV optical densities as indicators of antibody levels. Farm was included as a random effect, MAP serum data was included as a covariate, and MAP fecal culture, MAP PCR data and farm size data were included as classification variables. These models were used to assess associations in all the animals as a whole, as well as only those animals that tested positive for SRLV.

The overall apparent prevalence of SRLV, based on serology, in the 355 Ontario dairy sheep was 41.3% (95% CI: 36.3 to 46.5). Of the 19 farms sampled, 3 farms had a within flock prevalence of 100% and 3 farms had a prevalence of 0% (Figure 1). Flock-level prevalence was 78.9% (95% CI: 56.1 to 92.1) with 15/19 farms having at least 1 infected sheep. The prevalence of co-infection of SRLV and MAP in Ontario sheep was estimated at 3.4% (95% CI: 1.9 to 5.9) and 36.8% (95% CI: 19.1 to 59.1) of sampled farms had at least 1 animal test positive for both SRLV and MAP.

The seroprevalence of SRLV in the 558 sampled Ontario dairy goats was 80.1% (95% CI: 77.7 to 84.2). Of the 28 farms sampled, 3 farms had a prevalence of 0% and 11 farms had a within-herd prevalence of 100% (Figure 2). Additionally, 89.3% (95% CI: 71.9 to 97.1) or 25/28 of the farms sampled had at least 1 SRLV-infected goat. With respect to co-infection, 14.3% (95% CI: 11.6 to 17.5) of goats tested positive for SRLV and MAP, and 71.4% (95% CI: 52.8 to 84.9) or 20 of the 28 farms sampled had at least 1 co-infected animal. Additionally, 91.2% of MAP infected goats also tested positive for SRLV.

Analysis of the sheep SRLV data revealed no significant associations (\( P = 0.592 \)) (Table I); however, analysis of the goat SRLV data found a significant association between SRLV infection and MAP fecal culture (\( P = 0.021 \); Table II).

A cross-sectional study was done to assess the prevalence of SRLV infection in Ontario dairy sheep flocks and dairy goat herds, and to estimate the prevalence of SRLV and MAP co-infection in those same farms and animals. The seroprevalence of SRLV in Ontario dairy sheep was found to be 41.3% (95% CI: 36.3 to 46.5) and 78.9% (95% CI: 56.1 to 92.1) of the farms sampled had ≥ 1 infected animal suggesting that SRLV infection is common in Ontario dairy sheep flocks.

In Ontario dairy goats, the seroprevalence was 80.1% (95% CI: 77.7 to 84.2), and 89.3% (95% CI: 71.9 to 97.1) of study farms had ≥ 1 infected animal, which is in agreement with previous work that estimated a seroprevalence of SRLV in Ontario dairy goats to be 80.4% (6). This high prevalence of SRLV infection in both...
dairy sheep and dairy goats at the individual and farm level, suggests that SRLV may represent a serious problem to those industries, and emphasizes the need to implement preventative strategies into current farm management practices to prevent SRLV transmission.

This study also investigated SRLV and MAP co-infection in Ontario small ruminants. It is possible that the degree of immune dysregulation that occurs both during SRLV and MAP infections is exacerbated by co-infection. For example, HIV-1 and M. tuberculosis co-infected patients respond inappropriately to both pathogens, which can accelerate disease progression (13), and these altered immune responses may compromise the ability to detect both pathogens. Therefore, understanding how SLRV and MAP interact and how many animals are infected with both pathogens is important for assessing overall animal health. The apparent co-prevalence of SRLV and MAP was estimated at 3.4% (95% CI: 1.9 to 5.9) and 14.3% (95% CI: 11.6 to 17.5) in sheep and goats, respectively. Despite the high prevalence of both SRLV and widespread nature of MAP, the animal-level prevalence of co-infection was surprisingly low. However, the farm level prevalence was much higher with 36.8% (95% CI: 19.1 to 59.1) and 71.4% (95% CI: 52.8 to 84.9) of sheep and goat farms, respectively. Despite the high prevalence of both SRLV and widespread nature of MAP, the animal-level prevalence of co-infection was surprisingly low. However, the farm level prevalence was much higher with 36.8% (95% CI: 19.1 to 59.1) and 71.4% (95% CI: 52.8 to 84.9) of sheep and goat farms, respectively.

Although the animal level co-prevalence is low, it may be that the testing protocols used have underestimated the level of infection particularly in sheep. Detecting SRLV infection in these animals likely wasn’t an issue. Test sensitivity for the SRLV ELISAs are 99.4% and 100% in sheep and goats according to the manufacturers and since all animals sampled were over 2 y of age, animals infected at birth would have seroconverted (14). However, test sensitivity for MAP infection regardless of the diagnostic test used, has often been reported as moderate to low (15). The MAP test evaluations used in this study were previously conducted (8) and identified MAP fecal culture to be the most sensitive test in both sheep and goats. In goats, fecal culture is 81.1% (95% CI: 65.8 to 93.0) sensitive, while in sheep it is only 49.5% (95% CI: 27.4 to 72.5) sensitive. While culture still reported as moderate to low (15). Therefore, if MAP infection is underestimated, co-infection suggests that both MAP and SLRV are widespread in Ontario dairy sheep flocks and goat herds. This study further exemplifies the need for more producer education and implementation of on-farm SRLV control measures. These control measures include, removing newborn kids and lambs at birth and providing replacement colostrum and milk, limiting animal movements between farms, providing separate housing for animals of different ages, and routine testing and culling of infected animals (19). These prevention measures are also effective for preventing the spread of MAP and other pathogens, and will thus help ensure a healthy flock or herd. Additionally, further studies should focus on understanding the immune responses to SRLV and MAP infection, and how the pathogens interact as a means of improving diagnostic testing and vaccine development.

This is the first study to estimate the prevalence of SRLV and MAP co-infection in Ontario dairy sheep flocks and goat herds. The apparent prevalence of 3.4% and 14.3% in sheep and goats, respectively, suggests that few animals are in fact co-infected, however, this is likely underestimated. The high flock and herd level prevalence for co-infection suggests that both MAP and SLRV are widespread in Ontario dairy sheep flocks and goat herds. This study further validates a high prevalence of SRLV in Ontario sheep and goats indicating the widespread nature of SRLV in Ontario. Therefore, the Ontario sheep and goat industries should introduce measures to provide replacement colostrum and milk, limiting animal movements between farms, providing separate housing for animals of different ages, and routine testing and culling of infected animals (19). These prevention measures are also effective for preventing the spread of MAP and other pathogens, and will thus help ensure a healthy flock or herd. Additionally, further studies should focus on understanding the immune responses to SRLV and MAP infection, and how the pathogens interact as a means of improving diagnostic testing and vaccine development.

In goats, a significant positive association between animals that tested positive for SRLV and animals positive for MAP by fecal culture was identified, which suggests that those animals that are SRLV positive were more likely to be MAP infected or shed more bacteria. This supports the hypothesis that those animals that are infected with one pathogen may not respond to further infections with an appropriate immune response. The animal would then shed more bacteria or fail to absorb the bacteria and, therefore, it passes through into the feces. However, this association may also indicate that co-infected animals experience enhanced MAP growth within the small intestine and therefore shed more. Similar results have been found with HIV-1 and M. tuberculosis co-infection in human macrophages, where an increase in M. tuberculosis replication was noted in co-infected cells (18). This may also suggest that infection with SRLV may increase susceptibility to other infections, as is the case with HIV-1 patients (13). However, it is currently unknown how SRLV infection affects MAP replication and disease progression.

Further research is required to accurately identify animals that are co-infected with MAP and SRLV. Future studies should increase the number of farms sampled and collect more than one fecal sample from each animal to help identify those that may not be shedding the bacteria during the initial sampling period. The possibility of accelerated disease progression should also be investigated by monitoring the time to SRLV seroconversion in herds and flocks that are positive for both pathogens. The high prevalence of SRLV, further exemplifies the need for more producer education and implementation of on-farm SRLV control measures. These control measures include, removing newborn kids and lambs at birth and providing replacement colostrum and milk, limiting animal movements between farms, providing separate housing for animals of different ages, and routine testing and culling of infected animals (19). These prevention measures are also effective for preventing the spread of MAP and other pathogens, and will thus help ensure a healthy flock or herd. Additionally, further studies should focus on understanding the immune responses to SRLV and MAP infection, and how the pathogens interact as a means of improving diagnostic testing and vaccine development.

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Acknowledgments

This study failed to reveal any significant associations between the different MAP test results and SRLV infection in sheep. One possible reason for the lack of an association in sheep may be attributed to the ovine MAP test sensitivity as previously discussed; however, the small sample size in this study was also a limitation. Additionally, this study was a cross-sectional point prevalence study, so several animals may have been missed at the time of sampling due to intermittent shedding of MAP (17) or fluctuating SLRV antibody levels (14). Thus a cohort study may be more effective to track disease progression in co-infected animals over time.
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